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(54) Title: COMPOSITIONS FOR DELAYED TREATMENT OF ISCHEMIA-RELATED NEURONAL DAMAGE

(57) Abstract

Methods and compositions for reducing neuronal damage related to an ischemic condition in a mammalian subject are described. The method includes administration of compositions of the invention to the subject, 4-24 hours after the onset of the ischemic condition. Compositions of the invention are effective to selectively bind omega conotoxin binding sites, and preferably to bind with high affinity to omega conotoxin MVIIA binding sites, and to selectively block neurotransmitter release from mammalian CNS neuronal cells. Also disclosed are novel peptide structures useful in the treatment method of the invention.

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**COMPOSITIONS FOR DELAYED TREATMENT OF
ISCHEMIA-RELATED NEURONAL DAMAGE**

5 Field of the Invention

The present invention relates to pharmaceutical compositions and methods for reducing neuronal damage with an ischemic condition, such as stroke, and for methods of screening test compounds for inclusion in such compositions and methods.

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Background of the Invention

Ischemic damage to the central nervous system (CNS) may result from either global or focal ischemic conditions. Global ischemia occurs under conditions 5 in which blood flow to the entire brain ceases for a period of time, such as may result from cardiac arrest. Focal ischemia occurs under conditions in which a portion of the brain is deprived of its normal blood supply, such as may result from thrombo- 10 embolic occlusion of a cerebral vessel, traumatic head injury, edema, or brain tumors.

Both global and focal ischemic conditions have the potential for producing widespread neuronal damage, even if the global ischemic condition is 15 transient or the focal condition affects a very limited area.

Although these conditions appear to have similar underlying biochemical sequelae, the time scale over which they produce their respective damage may vary. 20 Thus, in global ischemia, in which cessation of blood flow is transient, though some permanent neuronal injury may occur in the initial minutes following cessation of blood flow to the brain, much of the damage appears several days following the ischemic event. Moreover, certain regions of the brain are 25 selectively vulnerable to the effects of global ischemia (Kirino, Pulsinelli (1982)). Secondary consequences of reperfusion of the tissue, such as the release of vasoactive products by damaged endothelium, and the release of cytotoxic products (free radicals, leukotrienes, etc.) by damaged tissues have been hypothesized to underlie the observed delay in neuronal damage.

30 Focal ischemia, on the other hand, may be of limited or prolonged duration. In the case of

prolonged focal ischemia, as caused by lodgement of a thromboembolus in a cerebral blood vessel, reduction of blood flow to a defined, focal region may be followed by reperfusion to part of the ischemic 5 region, via collateral circulatory pathways.

Ischemic cell death following focal ischemia has been reported to be complete 24 hours after the primary ischemic event (Nedergaard, 1987).

Several drug strategies have been proposed for 10 treatment of stroke and other neuronal conditions related to ischemia, and these have been reviewed in recent articles (e.g., Goldberg, Wauquier). Anti-coagulants, such as heparin, have been examined, but with mixed results. Similarly, antivasoconstriction 15 agents, such as flunarizine, excitatory neurotransmitter antagonists, such as MK-801 and AP7, and anti-edemic compounds have shown mixed results, with no clear benefits to outweigh a variety of side effects, including neurotoxicity or increased susceptibility 20 to infection.

Two general classes of vasodilators have been studied for possible treatment of neuronal ischemic damage. Non-specific vasodilators, including papaverine, prostacyclin, pentoxifylline, and 25 nitroprusside failed to demonstrate any clear benefit in reducing ischemic damage. A second general class of vasodilators includes a variety of calcium-antagonist vasodilator drugs. Verapamil and related compounds which prevent calcium entry into smooth and 30 striated muscle appear to be effective only at high drug concentrations, where serious cardiotoxicity effects may ensue. Dihydropyridines, such as nimodipine, have produced mixed results -- some 35 neurological improvement may be seen, but increased cerebral edema has also been observed.

Benzothiazepines, as exemplified by diltiazem, have shown moderate protective effects, but these drugs also appear to cause undesired side effects.

In general, the drugs mentioned above have been 5 administered prior to or within a few hours of the period of experimental ischemic insult. In clinical practice, particularly in the treatment of stroke, treatment is generally not feasible until well after the ischemic insult. In those studies in which 10 post-ischemia treatment has been given, the treatment paradigms have generally included treatment commencing before the ischemic event and continuing over an extended period of time, such as continuous administration of nimodipine from one hour before 15 until 24 hours following ischemia (Jacewicz), or repeated doses administered before as well as after the ischemic event (Dirnagl, 1990; Bielenberg, 1990). In one study, the NMDA antagonist MK-801 was 20 administered to Mongolian gerbils 24 hours post-ischemia, and neuroprotection was observed (Gill et al., 1988); however, the effects of this compound have subsequently been shown to be a consequence of postischemic hypothermia rather than a direct action 25 on NMDA receptors in this animal model (Buchan and Pulsinelli, 1990).

In summary, drugs which have been proposed to date for the treatment of stroke and other ischemic-related conditions of the brain are either (i) relatively ineffective, (ii) effective only at dosage 30 levels where undesired side effects are observed, and/or (iii) effective only when administered prior to or shortly after the ischemic insult.

In the parent U.S. Patent No. 5,051,403 and co-pending allowed patent application USSN 561,766, 35 filed November 22, 1989, and August 2, 1990,

respectively, and in applicants' PCT Application WO 91/079 the applicants have disclosed that omega-conotoxin peptides and related peptides which exhibit binding and N- or omega-type calcium channel 5 inhibitory properties similar to those of omega-conotoxin peptides are useful in reducing neuronal damage related to ischemic conditions. In the above-referenced applications, all of which are incorporated herein by reference, experiments 10 attesting to the efficacy of these compounds were conducted in accordance with standard experimental paradigms for examining neuroprotection. That is, test compounds were administered at the time of or up to 1 hour following the experimentally induced 15 occlusion which caused the ischemic event. In the current application, the applicants show that reduction of neuronal damage can be enhanced when the N-channel blocking compound is administered between ***4-24 hours following ischemia, relative to 20 immediate post-ischemia drug administration.

The applicants now show that compounds which are effective in reducing neuronal damage associated with ischemia are characterized by (a) relatively high affinity binding to an OCT binding site to which SNX-111 selectively binds (OCT site 1), and (b) relatively low affinity to a binding site selectively bound by OCT MVIIC (SNX-230) and OCT SVIB (SNX-183) (OCT site 2) in a synaptosomal preparation. This selective binding to OCT site 1 provides a basis for 25 great screening test compounds in a screening method 30 to select compounds for use in the treatment of cerebral ischemia.

35 Summary of the Invention

It is therefore a general object of the invention to provide a method for reducing neuronal damage related to an ischemic injury in the brain. This method is an improvement in the treatment 5 methods disclosed in co-owned PCT application WO 91/079.

The improvement is based on the discovery herein that compounds effective in reducing neuronal damage associated with ischemia are characterized by (a) 10 relatively high affinity binding to the SNX-111 synaptosomal binding site, and (b) relatively low affinity to an OCT-MVIIIC (SNX-230) binding site in a synaptosomal preparation. Further, in accordance with the present invention, it has been discovered 15 that such compounds can be administered hours after ischemic insult to a mammalian subject.

The method includes administering to the subject, at a time 4-24 hours following the onset of the ischemic condition, a compound which selectively 20 binds to an OCT MVIIA binding site in neuronal tissue.

Selectivity of binding is evidenced by a selectivity ratio of binding of the compound for the OCT MVIIA site, as compared to the affinity of the 25 compound in binding the OCT MVIIIC site, as described herein. Compounds are useful in the treatment method if they have a selective binding ratio to these two sites (MVIIA/MVIIIC) of at least 100, and preferably at least 500. More generally, effective compounds 30 exhibit a selectivity ratio of binding for the MVIIA site which is at least as great as that of an omega conotoxin selected from the group consisting of MVIIA, MVIIB, GVIA, GVIIA and RVIA omega conotoxins.

In a preferred embodiment, compounds useful in 35 the treatment method exhibit a high affinity for the

MVIIA binding site. Such high affinity is defined as an affinity which is at least as great as that of an omega conotoxin selected from the group consisting of MVIIA, MVIIB, GVIA, GVIIA and RVIA omega conotoxins.

5 In another embodiment, compounds useful in the treatment method are characterized further by their ability to selectively inhibit N-type voltage-gated calcium currents in neuronal tissue. In yet another embodiment, compounds useful in the treatment method
10 are characterized by their ability to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, as evidenced by a specific activity. Generally, active compounds will exhibit activities within the range of activities of MVIIA,
15 MVIIB, GVIA, GVIIA and RVIA omega conotoxins. In another embodiment, compounds are omega conotoxin compounds; in still another embodiment, such omega conotoxin compounds are selected from the group consisting of OCT MVIIA, OCT MVIIB, OCT GVIA, OCT
20 GVIIA, OCT RVIA, and SNX-207.

In another aspect, the invention includes a method of screening compounds for use in reducing ischemia-related neuronal damage, such as produced by stroke, in a human subject.

25 In the screening method of the invention, test compounds are assayed for their binding affinities to OCT-MVIIA and OCT-MVVIC binding sites in neuronal tissue, to determine a selectivity ratio of binding for the MVIIA site with respect to the MVIIC site.
30 The compound is selected if its selectivity ratio of binding for the MVIIA site is at least 100, and preferably, at least 500. More generally, a compound is selected, if its selectivity ratio of binding is at least as great as that of one of the omega
35 conotoxins MVIIA, MVIIB, GVIA, GVIIA or RVIA.

Test compounds can be further screened for their ability to selectively inhibit N-type voltage-gated calcium currents in neuronal tissue, again selecting those test compounds if their specific 5 activity, in producing such inhibition of calcium gated current, is at least as great as that of one of the omega conotoxins MVIIA, MVIIIB, GVIA, GVIIA or RVIA.

Test compounds can be further tested for their 10 ability to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, again selecting those test compounds if their specific activity, in producing such inhibition of neurotransmitter release, is at least as great as 15 that of one of the omega conotoxins MVIIA, MVIIIB, GVIA, GVIIA or RVIA.

In another embodiment, the test compound is an an omega conotoxin.

In yet another aspect, the invention includes 20 neuroprotective omega conotoxin peptides having the form: SEQ ID NO: 27-X₁-SEQ ID NO: 31-X₂X₃X₄-SEQ ID NO: 32-X₅X₆X₇-SEQ ID NO: 27-X₈-t, where X₁=K or L; X₂=X or R; X₃=T or L; X₄=S or M; X₅=T or S; X₆=K or R; X₇=R or K; and X₈=Y or R; and t= a carboxy or amidated 25 carboxyterminal group, excluding the peptides in which X₂=X, X₃=T, and X₄=S.

The invention further includes the 30 neuroprotective peptide SNX-207, having the structure SEQ ID NO: 20, wherein t= a carboxy or amidated carboxyterminal group. These neuroprotective peptides are also useful in the method, described herein, for treatment and reduction of ischemia-related neuronal damage in a mammalian subject.

These and other objects and features of the 35 invention will become more fully apparent when the

following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Figures

5 Figure 1 shows primary sequences of several natural omega-conopeptides, MVIIA/SNX-111 (SEQ ID NO: 01), MVIIIB/SNX-159 (SEQ ID NO: 02), GVIA/SNX-124 (SEQ ID NO: 03), GVIIA/SNX-178 (SEQ ID NO: 04), RVIA/SNX-182 (SEQ ID NO: 05), MVIID/SNX-238 (SEQ ID NO: 24), 10 SVIA/SNX-157 (SEQ ID NO: 06), TVIA/SNX-185 (SEQ ID NO: 07), SVIB/SNX-183 (SEQ ID NO: 08), MVIIIC/SNX-230 (SEQ ID NO: 21) and SNX-231 (SEQ ID NO: 22);

15 Figure 2 shows several analog omega-conopeptides SNX-190 (SEQ ID NO: 09), SNX-191 (SEQ ID NO: 10), SNX-193 (SEQ ID NO: 11), SNX-194 (SEQ ID NO: 12), SNX-195 (SEQ ID NO: 13), SNX-196 (SEQ ID NO: 14), SNX-197 (SEQ ID NO: 15), SNX-198 (SEQ ID NO: 16), SNX-200 (SEQ ID NO: 17), SNX-201 (SEQ ID NO: 18), SNX-202 (SEQ ID NO: 19), SNX-207 (SEQ ID NO: 20), 20 SNX-260 (SEQ ID NO: 23), and SNX-236 (SEQ ID NO: 25) and their relationships to SNX-111 (SEQ ID NO: 01), SNX-185 (SEQ ID NO: 07) or SNX-183 (SEQ ID NO: 08);

25 Figure 3A shows voltage-gated calcium current traces induced by a voltage step from -100 or -80 mV to -20 mV in untreated N1E-115 neuroblastoma cells (curve a) and in neuroblastoma cells exposed to increasing concentrations of OCT MVIIA (SNX-111) (curves b-d);

30 Figure 3B plots the percent inhibition of peak inward calcium currents in neuroblastoma cells as a function of OCT MVIIA (SNX-111) (solid triangles) and OCT GVIA (SNX-124) (solid circles);

35 Figure 4A shows voltage-gated calcium current traces induced by a voltage step from -70 to -20 mV in human neuroblastoma cells (IMR-32) in the absence

(lower trace) and presence (upper tracing) of 150 nM SNX-111;

Figures 4B and 4C show plots of absolute values of peak inward current measured every 15 seconds in 5 IMR-32 cells, elicited by pulses from -70 to 0 or -10 mV, versus time, where addition of compounds SNX-111 (4B) or SNX-111, SNX-183 (4C), and cadmium to the bathing medium are indicated by hatch marks just above the ordinate;

10 Figure 5A shows the inhibition of norepinephrine release from neuronal cells as a function of OCT MVIIA (SNX-111) concentration (solid bars are potassium stimulated and open bars are basal values);

15 Figure 5B shows a plot of evoked norepinephrine release from neuronal tissue as a function of concentration of OCT peptides SNX-230, SNX-111 or SNX-183;

20 Figures 6A and 6B are a binding curve showing the amount of OCT MVIIA (SNX-111) bound to rat synaptosomal membranes, as a function of OCT MVIIA (SNX-111) concentration (6A), and the same data plotted as a Scatchard plot (6B);

25 Figures 7A and 7B show plots of displacement of [¹²⁵I]SNX-111 (7A) and [¹²⁵I]SNX-230 (7B) by various OCT peptides;

30 Figures 8A and 8B show autoradiographs of binding of [¹²⁵I]SNX-111 (8A) and [¹²⁵I]SNX-183 (8B) to neuronal proteins separated by SDS polyacrylamide gel electrophoresis in the absence or presence of competing unlabeled ligand, as indicated;

35 Figures 8C and 8D show autoradiographs of binding of [¹²⁵I]SNX-111 (8C) and [¹²⁵I]SNX-230 (8D) to neuronal proteins separated by SDS polyacrylamide gel electrophoresis in the absence or presence of competing unlabeled ligand, as indicated;

Figures 9A and 9B show plots of displacement of binding of [¹²⁵I]SNX-111 (9A) and [¹²⁵I]SNX-183 (9B) by unlabeled OCT peptides SNX-111 and SNX-183 to 210 kilodalton proteins as described in the protein binding assay depicted in Figures 8A and 8B;

Figure 10A-10B are low-power micrographs of gerbil hippocampus CA1 region in animals after ischemia, and infusion of OCT MVIIA (SNX-111) (10A) or after ischemia and infusion of drug vehicle (10B);

Figures 11A-11D are higher power micrographs of cells in the drug-treated ischemic animals (11A, 11C, 11D), in animals receiving vehicle alone (11B), in animals showing complete protection by OCT against ischemic cell damage (11C); and in animals showing partial protection by OCT against ischemic cell damage (11D);

Figures 12A-12H show autoradiographs of coronal sections of rat brain to which is bound radiolabeled SNX-111 or SNX-183, as indicated, in the absence or presence of unlabeled peptide;

Figures 13A-13D show autoradiographs of sagittal sections of rat brain to which is bound radiolabeled SNX-111 or SNX-183, as indicated, in the absence or presence of unlabeled peptide;

Figure 14 shows amino acid sequences of active (groups I and II) and inactive (group III) OCT peptides and conserved peptide sequences within group I, Region a (SEQ ID NO: 26), Region b (SEQ ID NO: 27), Region c (SEQ ID NO: 28), Region d (SEQ ID NO: 29), Region e (SEQ ID NO: 28), and Region f (SEQ ID NO: 30), and within group II, Region s (SEQ ID NO: 27), Region t (SEQ ID NO: 31), Region u (SEQ ID NO: 32) and Region v (SEQ ID NO: 27);

Figure 15 shows a plot of hippocampal damage (CA1 region) as a function of dose of SNX-111

administered 1 hour (triangles) or 6 hours (circles) post-ischemia;

Figure 16 shows a plot of hippocampal damage as a function of time post-ischemia of administration of 5 a constant dose of SNX-111;

Figure 17 shows a bar graph of hippocampal damage at various doses of SNX-159 and SNX-111; and

Figure 18 shows a bar graph of hippocampal damage as observed 5 days and 12 days post-ischemia.

10

Detailed Description of the Invention

I. OCT Peptides

Omega-conotoxin (OCT) peptides are peptide toxins produced by marine snails of the genus Conus, and which act as calcium channel blockers (Gray). About 500 species of cone snails in the Conus genus have been identified, and a variety of OCT peptides from several of these species have been isolated. Omega-conotoxin peptides are alternatively referred to as "OCT peptides" or "omega-conopeptides" herein. The primary sequences of eight natural OCT peptides are shown in Figure 1. Conventional letter initials are used for the amino acid residues, and X represents 4-hydroxyproline, also abbreviated 4Hyp. All of the peptides shown in the figure are amidated at their C-termini.

The peptides shown in Figure 1 are identified by names which are commonly associated with either the naturally occurring peptide (single letter followed by a Roman numeral followed by a single letter), and by a synthetic designation (SNX-plus numeral). Either or both of these designations will be used interchangeably throughout the specification. For example, the peptide whose sequence is designated 35 MVIIA/SNX-111 will be referred to herein as OCT

MVIIA, or alternatively, SNX-111, the latter to signify that the compound is synthetic in origin. Synthetic and naturally occurring peptides having the same sequence behave substantially identically in the assays and methods of treatment of the invention. The OCT MVIIA (SNX-111) and OCT GVIA (SNX-124) peptides also have the common names CmTx and CgTx, respectively. All of the OCT peptides have three disulfide linkages connecting cysteine residues 1 and 4, 2 and 5, and 3 and 6, as indicated for the MVIIA peptide in Figure 2.

Figure 2 shows analogs of natural OCT MVIIA, OCT TVIA, and OCT SVIB peptides which have been synthesized and tested in accordance with the invention. Standard single amino acid code letters are used in the figure; X=hydroxyproline; Nle=norleucine; NH₂ group at the C terminus indicates that the peptide is C-terminal amidated; G-OH indicates termination in an unmodified glycine residue.

A. Preparation of OCT Peptides

This section describes the synthesis, by solid phase methods, of several naturally occurring omega conotoxin (OCT) peptides and additional OCT peptides which are used in the present invention.

OCT peptides, such as those shown in Figures 1 and 2, can be synthesized by conventional solid phase methods, such as that detailed in U.S. Patent No. 5,051,403, and PCT patent application WO 91/079, both of which are incorporated herein by reference.

These methods are detailed in Example 1 herein, for the synthesis of exemplary OCT MVIIC/SNX-230.

35 II. In vitro Properties of OCT Peptides

This section describes *in vitro* properties of OCT peptides. Generally, these properties are described for neuroprotective OCT peptides. For purposes of comparison, properties of OCT MVIIC/SNX-5 230, a peptide lacking neuroprotective activity, are also described and distinguished in this section.

A. Calcium-Channel Antagonist Activity

10 The neuroprotective compounds of the invention are neuronal-cell calcium channel antagonists, as defined by their ability to inhibit voltage-gated ionic currents in neuronal cells.

Voltage-gated calcium channels are present in 15 neurons, and in cardiac, smooth, and skeletal muscle and other excitable cells, and are known to play a variety of roles in membrane excitability, muscle contraction, and cellular secretion, such as in synaptic transmission (McCleskey). In neuronal cells, voltage-gated calcium channels have been 20 classified by their electrophysiological as well as by their biochemical (binding) properties.

Electrophysiologically, these channels can be classified either as Low-voltage-activated (LVA) or High-voltage-activated (HVA). HVA channels are 25 currently known to comprise at least three groups of channels, known as L-, N- and P-type channels (Nowycky, Sher). These channels can be distinguished electrophysiologically as well as biochemically on the basis of their pharmacology and ligand binding 30 properties. Thus, dihydropyridines, diphenylalkylamines and piperidines bind to the alpha, subunit of the L-type calcium channel and block a proportion of HVA calcium currents in neuronal tissue, which are termed L-type calcium 35 currents.

Omega conotoxins also block a proportion of HVA calcium currents in neuronal tissue, and, in the presence of a maximally inhibitory quantity of dihydropyridine compound, effect substantially 5 complete inhibition the remaining HVA currents in neuronal cells. These calcium currents are identified as N-type calcium currents, though recently a proposal that such currents be termed "omega" has been presented (Sher). Omega conotoxins 10 bind to a specific population of binding sites. Dihydropyridines and other L-type channel blockers do not displace omega conotoxin binding, nor do omega conotoxins displace binding of ligands to L-channels. Unlike L-type calcium channels, omega channels are 15 found predominantly, although not exclusively, in nervous tissue (Sher).

One suitable system for testing inhibition (blockage) of N-type or omega HVA neuronal calcium channels is an isolated cell system, such as the 20 mouse neuroblastoma cell line, strain N1E115 or the human neuroblastoma cell line IMR32. Membrane currents are conveniently measured with the whole cell configuration of the patch clamp method, according to the procedure detailed in Example 2. 25 Briefly, a voltage clamp protocol was performed in which the cell potential was stepped from the holding potential of about -100 mV to test potentials that ranged from -60 mV to +20 mV, and the cell was held at the holding potential for 5 seconds between 30 pulses.

Figure 3A shows a typical inward calcium current in an N1E115 neuroblastoma cell elicited by a voltage step from -80 mV to -20 mV in the absence of OCT, as 35 detailed in Example 2. In this, and most of the recordings shown, barium (Ba) replaced calcium (Ca)

as the charge-carrier through the calcium channels in order to increase the signal (McCleskey). As seen from Figure 3A, curve a, the calcium current activates quickly (within about 20 ms) and 5 inactivates with a time constant of 30 to 40 ms. The calcium current is measured by the amplitude of the peak inward current elicited by the depolarization peak, and has a measured value of about -1200 pA. The cell in Figure 3A (curve a) was also exposed to 10 μ M nifedipine, a dihydropyridine, which is expected to effectively block L-type calcium channels in the neuroblastoma cells, and no effect on the measured calcium current was observed. The calcium current observed is thus not dihydropyridine-sensitive. The 15 responses of voltage-gated calcium current to increasing concentrations of OCTs MVIIA (SNX-111) and GVIA (SNX-124) are shown in Figure 3B. The ED_{50} concentration, at which 50% inhibition of calcium current is produced, is determined from the voltage-gated current amplitudes, plotted as a function of OCT peptide concentration. The calculated ED_{50} is about 10 nM for GVIA and 100 nM for MVIIA, indicative of high inhibitory peptide activity. The ED_{50} concentration for these and OCT peptides SVIA (SNX-20 157) and SVIB (SNX-183) are given in Table 1 below. The two compounds with relatively low IC_{50} values (below 1 μ M) are both active as neuroprotective 25 agents, as will be seen in Section III below, whereas the OCT SVIA and SVIB peptides with IC_{50} values above this threshold are not. More generally, the 30 compounds of the invention are classified as antagonists of voltage-gated calcium channels by their ability to inhibit voltage-gated calcium channel currents characterized as above with an ED_{50}

value of less than about 1 μ M in the assay detailed in Example 2.

Table 1

Inhibition of calcium currents in N1E-115 neuroblastoma cells

	Compound	<u>IC₅₀</u>
10	GVIA (SNX-124)	10 nM
	MVIIA (SNX-111)	100 nM
	SVIB (SNX-183)	> 1 μ M
	SVIA (SNX-157)	>20 μ M

15 Calcium currents were also measured in human neuroblastoma IMR32 cells, using techniques described above and in Example 2. Voltage-gated calcium currents were elicited by holding the cell(s) at -70 mV and administering a step-voltage to -10 mV. 20 Current tracings from IMR-32 cells bathed in control medium (lower curve) and in medium containing 150 nM SNX-111 (upper curve) are shown in Figure 4A. In this experiment attenuation of voltage-gated calcium 25 current is apparent in the presence of SNX-111 (upper curve), as shown by the decreased amplitude of the peak inward current.

30 Figures 4B and 4C show cumulative data from many consecutive currents, elicited at 15 second intervals as described above, in IMR-32 cells. In these plots, peak inward current recorded from each stimulus is recorded sequentially as a single data point. In the 35 experiment illustrated in Figure 4B, addition of SNX-111 to the bathing medium resulted in decreased peak inward currents; restoration of substantially normal

calcium currents was achieved after washing of the compound from the cell chamber, shown on the right side of the figure. Figure 4C shows the effects of 150 nM SNX-111 and SNX-183 added sequentially to a 5 single cell preparation. Both compounds resulted in attenuation of peak inward current; though recovery following SNX-183 exposure was not observed. Addition of cadmium (Cd) to the medium resulted in blockade of all remaining voltage-gated calcium 10 currents in this preparation.

Test peptides which are inhibitory for neuronal cell calcium currents can be further tested in non-neuronal cells, to confirm that the peptide activity in blocking calcium currents is specific to neuronal 15 cells. A variety of muscle cell types which are refractory to calcium-current inhibition by OCTs, such as vertebrate embryo heart and skeletal muscle cells, are suitable. Cell current measurements are made substantially as outlined above and detailed in 20 Example 2. OCT MVIIA, for example, has been reported to block voltage-gated calcium channels in a variety of neuronal cells, including dorsal root ganglion (DRG) neurons (McCleskey). This blockage or inhibition of calcium channel currents has been 25 reported to be neuron-specific, since calcium current inhibition by the peptide was not observed in cardiac, smooth, and skeletal muscles.

OCT MVIIIC, a non-neuroprotective OCT peptide also inhibits certain calcium currents. However, 30 these currents, like the OCT peptides described above, OCT MVIIIC (SNX-230) has no effect on dehydropyridine-sensitive currents. This compound exhibits a distinctive electrophysiological profile which includes blockade of a significant fraction of 35 dehydropyridine-resistant, OCT GVIA-resistant (non-L,

non-N type) current in hippocampal CA1 neuronal cells, as well as inhibition of high threshold calcium channel current in cerebella Purkinje neurons; as described in co-owned U.S. Patent 5 Application 916,478 and subsequently published (Hillyard, et al.). Unlike OCT GIVA, OCT MVIIIC additionally blocks calcium uptake in rat brain synaptosomes at low (2.5 μ M) concentration. Unlike MVIIA, it also shows high potency in inhibition of phrenic nerve-mediated muscle concentrations in an 10 isolated mouse diaphragm preparation.

B. Selective Inhibition of Norepinephrine Release

A second requisite property of neuroprotective 15 compounds, in accordance with the invention, is the ability to specifically inhibit depolarization-evoked and calcium-dependent norepinephrine release in brain (CNS) neuronal cells, but not inhibit neurotransmitter release at a mammalian neuromuscular junction of a skeletal muscle. Inhibition of 20 norepinephrine release in neuronal cells can be assayed in mammalian brain hippocampal slices by standard methods, such as detailed in Example 3.

Figure 5A shows effects of increasing 25 concentrations of OCT MVIIA peptide on norepinephrine release from rat brain hippocampal slices which were first bathed in normal wash solution (open bars), then stimulation medium (solid bars). As seen, the compound produces a strong concentration-dependent 30 inhibition of norepinephrine release in the presence, but not in the absence of stimulation medium. From the concentration-dependent inhibition data, the compound concentration effective to produce 50% inhibition of norepinephrine release (IC_{50}) is 35 calculated.

Figure 5B shows a comparison of the effects of the three OCT peptides, MVIIA/SNX-111, SVIB/SNX-183, and MVIIIC/SNX-230, on the release of norepinephrine evoked by potassium depolarization *in vitro*, as detailed in Example 3. SNX-111 inhibits release with high potency (IC_{50} = ~ 1 nM) but only partially (~60%). SNX-183 is much less potent (IC_{50} = 180-200 nM) but the inhibition is virtually complete. SNX-230 also inhibits release 100% but in a biphasic manner, inhibiting approximately 50% with high potency (IC_{50} = 0.02 nM) and 50% with much lower potency (IC_{50} = 65 nM). These results imply that norepinephrine release is mediated by at least two distinct OCT-sensitive calcium channels, one of which corresponds to the site 1 receptor identified by high affinity for SNX-111 and the other to the site 2 receptor recognized preferentially by SNX-230, as described below.

The IC_{50} values given in Table 2 for a variety of OCT peptides which have been examined by this method represent the average IC_{50} values calculated from thin (200 μ) and thick (400 μ) hippocampal slices. The three lowest IC_{50} values, between 0.8 and 2.4 nM, correspond to OCT peptides which show pronounced neuroprotective activity (Section III below). The OCT peptides SNX-195 and SNX-201 are OCT MVIIA with amino acid substitutions or modifications at key residue sites (Figure 2), as will be discussed in Section IV below. The higher IC_{50} values measured for these modified peptides is reflected in substantial reduction or loss of neuroprotective activity. Peptides SVIA (SNX-157) and SVIB (SNX-183) are representative of OCT compounds which show no neuroprotective activity, and this is reflected by

high IC_{50} values for norepinephrine release. The SNX-202 peptide is a modification of SVIB peptide in which the Ser-Arg-Leu-Met residues at positions 9-12 in OCT MVIIA (SNX-111) are substituted for the Arg-Lys-Thr-Ser residues at the same positions in OCT SVIB (SNX-183). This modification significantly reduced the IC_{50} value for inhibition of norepinephrine release, but neuroprotective activity was not observed at a dose (2 μ g ICV) at which SNX-111 generally provided neuroprotection. These modifications were also reflected in binding specificity of the compounds to OCT sites 1 and 2, as discussed in Part C, below.

15

Table 2

Inhibition of Norepinephrine
Release by OCT Peptides

20	<u>OCT Peptides</u>	<u>IC_{50} (nM)</u>
	GVIA (SNX-124)	0.8
	MVIIA (SNX-111)	1.5
	TVIA (SNX-185)	2.4
	SNX-201	11
25	SNX-195	11
	SNX-202	29
	SVIB (SNX-183)	200
	SNX-191	>100
	SVIA (SNX-157)	>4500

30

In summary, pronounced neuroprotective activity is associated with an ability to inhibit norepinephrine release with an IC_{50} value which is within the range of IC_{50} values measured for active OCT peptides MVIIA (SNX-111), GVIA (SNX-124), and

TVIA (SNX-185); i.e., less than the largest of the IC₅₀ values measured for these active OCT peptides. However, with referenced to the activity of OCT MVIIIC, inhibition of NE release is not sufficient in 5 itself to predict neuroprotective activity.

C. Specific, High Affinity Binding to OCT Receptors

Another property of neuroprotective compounds, in accordance with the invention, is high-affinity 10 binding to an OCT MVIIA (SNX-111) binding site in neuronal cells. As will be seen below, the binding affinity can be characterized either by the binding constant of the compound for the MVIIA (SNX-111) binding site, or by the ratio of binding constants 15 measured for binding to neuronal-cell MVIIA-selective binding site (designated site 1) and SVIB/MVIIIC-selective binding site (designated site 2).

20 1. Binding of OCT Peptides to Neuronal Membranes

a. Binding to OCT site 1. Binding to OCT MVIIA binding site in neuronal tissue can be demonstrated with a variety of cell types and synaptosomal cell fractions. One preferred neuronal 25 membrane is a mammalian brain synaptosomal preparation, such as the rat brain synaptosome preparation described in Example 4. The binding constant of a compound for the MVIIA binding site is typically determined by competitive displacement of 30 radiolabeled OCT MVIIA (SNX-111) from the synaptosomal preparation, as follows.

The binding constant K_d of the MVIIA (SNX-111) peptide for the synaptosomal membranes is determined by a saturation binding method detailed in Example 35 5A. The plot of bound peptide as a function of

concentration is then used to calculate a B_{max} , the concentration of binding sites on the synaptosomes, and K_d following standard methods. In particular, the K_d value is the calculated concentration of peptide needed to half saturate the synaptosomal specific binding sites. Figure 6A shows the specific binding of radiolabeled OCT MVIIA (SNX-111) to rat brain synaptosomes, plotted as a function of OCT peptide concentration, and Figure 6B, the same data in Scatchard plot form. From the slope of the Scatchard plot line, a K_d binding value of 8.8 pM is obtained.

To determine the binding constant of a test compound for the MVIIA binding site, the test compound is added, at increasing concentrations to the synaptosome preparation having bound, radiolabeled OCT MVIIA. The synaptosomal material is then rapidly filtered, washed and assayed for bound radiolabel, as detailed in Example 5B. The binding constant (K_d) of the test compound is determined from computer-fit competitive binding curves, such as shown in Figure 7A for MVIIA (SNX-111) peptide, to determine first the IC_{50} value of the compound, i.e., the concentration which gives 50% displacement of labeled MVIIA peptide, then calculating K_i from the K_d value of OCT MVIIA and the IC_{50} value of the compound, as detailed in Example 5. IC_{50} values for a number of OCT peptides for inhibition of OCT MVIIA binding are shown in Table 3. The compounds are arranged in order of increasing IC_{50} values.

Table 3

Competition of ^{125}I -MVIIA (SNX-111)
Binding by OCT Peptides

		IC ₅₀ (nM)
	SNX-207	.007
	SNX-194	.008
	SNX-195	.009
5	MVIIA (SNX-111)	.013
	SNX-190	.021
	SNX-200	.039
	SNX-201	.046
	SNX-202	.046
10	SNX-193	.070
	MVIIIB (SNX-159)	.101
	GVIA (SNX-124)	.134
	SNX-198	.160
	SNX-191	.165
15	TVIA (SNX-185)	.228
	SNX-196	.426
	RVIA (SNX-182)	.893
	SVIB (SNX-183)	1.09
	GVIIA (SNX-178)	3.70
20	SNX-197	11.3
	SVIA (SNX-157)	1460.

Compounds with known neuroprotective activity,
25 such as SNX-207, OCT MVIIA (SNX-111), GVIA (SNX-124),
and TVIA (SNX-185), have IC₅₀ values between about 15
and 300 pM, in the assay shown, and corresponding K_i
values between about 1 and 100pM. Conversely, OCT
peptides, such as OCT SVIA (SNX-157) and SVIB (SNX-
183), which are not neuroprotective have
30 substantially greater IC₅₀ and K_i values for
displacement of OCT MVIIA binding.

A number of OCT peptide compounds which were tested gave IC_{50} and K_i values lower than or within the ranges of those of OCT peptides MVIIA (SNX-111), GVIA (SNX-124), and/or TVIA (SNX-185), and these 5 compounds should thus be considered candidates as neuroprotective compounds. However, some of these compounds, such as SNX-201, SNX-195, and SNX-202 have IC_{50} values for inhibition of norepinephrine release which are outside the range of neuroprotective compounds (Table 2), and thus these compounds do not 10 meet all of the criteria for neuroprotective compounds.

b. Binding to OCT Site 2. It has also 15 been discovered, in accordance with the invention, that compounds with high neuroprotective activity show relatively low binding affinity for a second OCT binding site. This site is defined by binding of radioiodinated OCT SVIB (SNX-183) or radioiodinated 20 OCT MVIIC (SNX-230) binding to neuronal membranes. Conversely, high binding affinity for this second site is observed with some inactive compounds. IC_{50} and K_i values for compound binding to this site are calculated, as above, by determining the K_d of 25 radioiodinated OCT SVIB (SNX-183) or of radiolabeled OCT MVIIC (SNX-230) for binding to a synaptosome preparation, then using competitive displacement of the labeled compound by the test compound, to determine the IC_{50} and K_i values of the test compound.

30 Saturation binding of [^{125}I]-SNX-230 to a rat brain synaptosomal preparation showed that the radiolabeled SNX-230 also displayed high affinity (apparent K_d = 0.012nM) but that the ranked order of 35 potencies for displacement of this binding by the same set of OCT peptides is very different from that

observed using [¹²⁵I]-SNX-111 as the radioligand. Figure 7B shows computer-fit competitive binding curves for several OCT peptides whose binding to the MVIIC (SNX-230) binding site was examined. From 5 these curves, IC₅₀ and K_i values were determined as above. Further analysis indicated that the relative potency of displacement by OCT peptides of SNX-230 binding was similar to that of radioiodinated SNX-183 binding. This difference in rank order of potencies 10 at the site labeled by SNX-111 and the site labeled by iodinated SNX-183/SNX-230 points to the existence of at least two distinct OCT peptide binding sites present in brain tissue. These sites are referred to as OCT site 1 and OCT site 2 herein.

15 Inhibitory constants (K_i's) for SNX-111 and SNX-230, calculated as detailed in Example 4 from the IC₅₀, shown Figure 7A and Figure 7B, are presented in Table 4. Whereas SNX-111 displayed the highest affinity (K_i = 0.002nM) for the OCT site 1, it was 20 found to have much lower affinity (K_i = 150nM) OCT site 2. The K_i values obtained for SNX-230 in the two binding assays show that this novel omega-conopeptide from *C. magus* is selective for site 25 2 over site 1 by a factor of seven (K_i = 0.03nM and 0.2nM, respectively). Another conopeptide, OCT SVIB/SNX-183, this one from *C. striatus*, displayed substantially lower affinity to both sites with a slight preference for site 1 (Table 4). As seen, 30 neuroprotective compounds OCT MVIIA (SNX-111), GVIA (SNX-124), SNX207 and TVIA (SNX-185) exhibited highest relative displacement potencies at the MVIIA site. Table 4 also shows ratios of relative 35 potencies of binding for each compound at the MVIIA (site 1) and MVIIC (site 2) binding sites. These ratios accentuate the difference in binding

properties between neuroprotective compounds, and those which show no neuroprotective activity within the range of concentrations tested.

5

Table 4

Compound	Ki (nM) for competition ^a with:		Selectivity ^b for:	
	[¹²⁵ I]-SNX-111	[¹²⁵ I]-SNX-230	site 1	site 2
SNX-111	0.003	200	67,000	: 1
SNX-124 ^c	0.009	315	35,000	: 1
SNX-157	500	>100,000		
SNX-159	0.03	14	470	: 1
SNX-178	1.3	ND ^d		
SNX-182	0.4	140	350	: 1
SNX-183	0.3	4	13	: 1
SNX-185	0.08	3,000	38,000	: 1
SNX-230	0.17	.02	1	: 8.5

20 *Ki values were derived from analysis of competitive binding performed as described in Example 5.

30 'The result for SNX-124 (GVIA) is an apparent Ki under the given experimental conditions. It is not possible to calculate a true Ki value since GVIA binds to its receptor irreversibly.

⁴ = not determined.

35

From the foregoing, it is seen that neuroprotective compounds in accordance with the invention are characterized by a high binding affinity for the MVIIA binding site, OCT site 1, on neuronal membranes. The binding affinity for this

site may be characterized in one of two ways. In the first approach, the binding affinity of the compound for the MVIIA site, as estimated by IC_{50} at the site, is compared directly with those of SNX-111, SNX-207, 5 SNX-124, or SNX-185. A neuroprotective compound is one whose binding affinity is at least as high as and preferably within the range of binding affinities measured for the OCT's MVIIA (SNX-111), GVIA (SNX-124), and TVIA (SNX-185), i.e., the binding constant 10 is no greater than the highest binding constant among these four OCT peptides.

Alternatively, the binding affinity for the MVIIA site (site 1) can be characterized by the ratio of binding constants or relative potencies for the 15 MVIIA-selective and SVIB/MVIIIC-selective (site 2) sites, as just described. Here a neuroprotective compound is one whose binding ratio is within the range of such binding ratios measured for the OCT's SNX-207, MVIIA (SNX-111), GVIA (SNX-124), and TVIA 20 (SNX-185), i.e., the binding ratio is no lower than the smallest ratio among these four OCT peptides.

To demonstrate the importance of specific regions of OCT peptides in conferring site selectivity, comparison of the amino acid sequences 25 of SNX-111 and SNX-183 (Figure 1) shows that these two conopeptides have a high degree of similarity in the amino terminal and carboxy terminal segments. Five of the first eight residues and 12 of the last 14 are identical in the two peptides, and the 30 greatest difference occurs in the four middle residues, 9 through 12. To assess whether or not this middle segment plays a role in determining the difference in selectivity of the two peptides, two hybrid molecules were synthesized by interchanging 35 this segment between SNX-111 and SNX-183. The hybrid

peptide SNX-201 is identical to SNX-111 except for residues 9-12 (-S-R-L-M-) which have been replaced by the corresponding residues (-R-K-T-S-) from SNX-183. Similarly, SNX-202 contains residues 9-12 from SNX-111 in place of the corresponding residues in the sequence of SNX-183.

Replacement of residues 9-12 in SNX-111 by the corresponding segment from SNX-183 (compound SNX-201) lowers the affinity for site 1 five-fold and 10 increases the affinity for site 2 by a factor of 12. Conversely, introduction of the segment 9-12 from SNX-111 into the corresponding region of SNX-183 (compound SNX-202) increases the affinity for site thirty-four-fold and lowers the affinity for site 2 four-fold. Thus, a significant portion of the 15 specific binding of the conopeptides to their receptor subtypes is due to the specific interaction of the middle segment with the receptor.

20 2. Binding to Specific Proteins in Neuronal Tissue

Conopeptide receptor polypeptides in rat brain or hippocampal synaptic membranes were chemically cross-linked to radio-iodinated SNX-111, SNX-183, or 25 SNX-230 with a water-soluble carbodiimide, as detailed in Example 6. The radiolabeled peptides were separated by SDS-PAGE and visualized by autoradiography. These studies, as described below, provided further evidence that the two receptor sites 30 identified by SNX-111 and SNX-230 are constituent parts of distinct calcium channels.

[¹²⁵I]-SNX-111, [¹²⁵I]-SNX-183, and [¹²⁵I]-SNX-230 were chemically crosslinked to synaptosomal membrane preparations and then subjected to SDS-PAGE followed 35 by autoradiography. With [¹²⁵I]-SNX-111, essentially

only one polypeptide band of M_r 210-220 kDa was specifically labeled. SNX-111 inhibited the incorporation of radioactive SNX-111 into this band with an IC_{50} of 0.03 nM, in good agreement with the 5 IC_{50} for site 1 determined by binding assays (Fig. 8A). Labeling of this 210-220 kDa band by [^{125}I]-SNX-111 is also inhibited by SNX-183 but with lower affinity (IC_{50} = 0.3 nM) as shown in Figure 9A. Similar experiments with [^{125}I]-SNX-183 as 10 crosslinking agent revealed that in addition to the expected labeled band at 210kDa, three additional bands at approximately 170, 150 and 140 kDa appear to be specifically labeled (Fig. 8B). Similar results are observed when crosslinked [^{125}I]-SNX-230 is 15 compared with cross-linked [^{125}I]-SNX-111 on gels. That is, SNX-230 binds specifically to the 210-220 band plus three additional protein bands which migrate as 170, 150 and 140 kDa (Figure 8C and Figure 8D). In this experiment, [^{125}I]SNX-230 was 20 incorporated at a concentration (0.1 nM) at which site 2, but not site 1 should be labeled, according to the equilibrium binding data shown above. Analysis of the inhibition of incorporation of [^{125}I]-SNX-183 into the 210-220 kDa band by SNX-111 25 provides good evidence for the presence of two distinct polypeptides of approximate M_r 210 kDa corresponding to site 1 and site 2 (Fig. 9B). That is, SNX-111 displaces [^{125}I]-SNX-183 from the 210 kDa polypeptide in a biphasic manner with IC_{50} values of 30 0.006 nM and 65 nM. At low concentrations, SNX-111 effectively competes for binding to site 1; the binding of [^{125}I]-SNX-183 to site 2 is competed out only at much higher concentrations of SNX-111 because of the poor affinity of SNX-111 for site 2.

Inhibition of incorporation of [¹²⁵I]-SNX-183 into the 210 kDa band by SNX-183 is consistent with the ability of this iodinated peptide to bind to both site 1 and site 2 with nearly equal affinity (Table 5). Thus, the displacement curve is shallow with an IC₅₀ of 0.36 nM. Competition for the lower molecular weight bands was monotonic for both SNX-111 and SNX-183, and occurred only at higher concentrations of both peptides.

10 The crosslinking experiments strongly suggest that the conopeptide binding components of site 1 and site 2 are distinct molecular entities, both having M, 210-220 kDa. Taken together, the binding studies, neurotransmitter release assays, and the crosslinking 15 experiments indicate that site 1 and site 2 represent distinct calcium channels.

3. Binding to Distinct Regions of the Central Nervous System

20 It is now well established that subtypes of various neurotransmitter receptors are expressed in a tissue-specific and region-specific manner. The regional distribution of the OCT peptide binding sites in rat brain sections were determined by 25 autoradiography, as detailed in Example 7. The results presented in Fig. 12 show that the distribution of binding of [¹²⁵I]-SNX-111 is highly localized (Figures 12A and 12B) and that non-specific binding is virtually non-existent (Figures 12C and 12D). Comparison of the specific binding of 30 [¹²⁵I]-SNX-111 and [¹²⁵I]-SNX-183 (shown in Figures 12E and 12F) revealed overlapping but differential distributions of binding sites. Both ligands labeled the cortex, CA1, dentate gyrus, and caudate-putamen. 35 In these regions, binding of [¹²⁵I]-SNX-183 was

unaffected by concentrations of SNX-111 that completely displace [¹²⁵I]-SNX-111 labeling, suggesting both a co-localization of sites 1 and 2 and a preponderance of [¹²⁵I]-SNX-183 labelled site 2 receptors in these regions. Greater abundance of site 2 channels in thalamic ventromedial lateral lobe and medial geniculate was revealed by the high density of binding of [¹²⁵I]-SNX-183 in these nuclei. In contrast, globus pallidus, CA2, and substantia nigra were labeled only by [¹²⁵I]-SNX-111, indicating a preponderance of site 1 channels in these regions.

The complete absence of [¹²⁵I]-SNX-183 binding in the substantia nigra (Figure 12F) which displays profuse [¹²⁵I]-SNX-111 binding, suggests a third, distinct binding site recognized only by [¹²⁵I]-SNX-111, and by implication, another novel calcium channel subtype. Likewise, since voltage-sensitive regulation of calcium influx is common in neuronal functioning, those regions of the brain lacking binding of SNX-183 or SNX-111 are likely to contain a different calcium channel subtype.

Site 1 and site 2 binding was also characterized by comparison of binding of [¹²⁵I]-SNX-111 and [¹²⁵I]-SNX-230 in sagittal sections of rat brain as shown in Figure 13(A-D). As shown, [¹²⁵I]-SNX-111 (Figure 13A) and [¹²⁵I]-SNX-230 (Figure 13B) bind differentially to various brain structures. Figures 13C and 13D show non-specific binding of [¹²⁵I]-SNX-111 and [¹²⁵I]-SNX-230, respectively, carried out in the presence of 25 mM uniodinated peptide.

III. Neuroprotective Compositions and Methods of Reducing Neuronal Damage Related to an Ischemic Condition

The present invention provides a method and composition of the invention effective to reduce neuronal damage related to an ischemic condition in a human patient. The ischemic conditions may be due to
5 an interruption in cerebral circulation, such as caused by cardiac failure, or other condition leading to global loss of blood supply to the brain, or to localized interruptions in blood flow, such as due to cerebral hemorrhage, or localized thrombotic or
10 embolic events, or head trauma.

The ischemic condition which is to be treated using the method and composition is generally associated with stroke, defined as the sudden diminution or loss of neurological function caused by an
15 obstruction or rupture of blood vessels in the brain, or by complete cessation of blood flow to brain, as in cardiac failure. In stroke, as well as in other types of cerebral ischemic conditions, the treatment method is aimed at preventing or reducing secondary
20 brain damage resulting from the original ischemic event. The secondary damage typically includes cerebral cell destruction, or lesions, in the area surrounding the ischemic injury, in the case of focal ischemia, and also in areas of selective vulnerability, such as the hippocampus or basal ganglia, in
25 the case of global ischemia. The secondary damage may often be manifested by functional impairment, such as loss of short-term or long-term memory. As will be seen below, the treatment method of the
30 invention is effective in reducing or preventing both anatomical and functional secondary damage related to ischemia.

Pharmaceutical compositions include a neuronal-cell calcium channel antagonist compound having
35 activities for selectively blocking norepinephrine

release in mammalian neuronal cells, and for binding to neuronal-membrane omega-conotoxin MVIIA binding site (OCT site 1), which are within the ranges of such activities for OCT peptides SNX-207, MVIIA (SNX-111), GVIA (SNX-124), or TVIA (SNX-185). The binding activities may be expressed either as binding constants for the MVIIA site on neuronal membranes, or as a ratio of the binding constants for the MVIIA and MVIIIC/SVIB binding sites (sites 1 and 2, respectively), as discussed in Section II above. The compound is carried in a suitable pharmaceutical carrier, such as a sterile injectable solution.

One exemplary class of neuronal cell calcium channel antagonists is that class of OCT peptides having the requisite inhibitory and binding activities. The peptide is formulated for parenteral administration in a suitable inert carrier, such as a sterile physiological saline solution. The concentration of peptide in the carrier solution is typically between about 0.1-20 mg/ml. The dose administered will be determined by route of administration. One suitable route is intracerebroventricular (ICV), at a dose level of about 0.1 to 20 μ g peptide/kg body weight, depending on the binding and inhibitory values of the peptide. The peptide compound may alternatively be administered intravenously (IV) as a bolus or as a continuous infusion as demonstrated below. It may be desirable for IV administration to pretreat the subject with antihistamines specific for H1 and H2 histamine receptors, to reduce possible blood pressure lowering after peptide administration.

In the parent U.S. Patent No. 5,051,403 and co-pending patent application USSN 561,766, filed

November 22, 1989, and August 2, 1990, respectively, and in related PCT application WO 91/079, the applicants have disclosed that omega-conotoxin peptides and related peptides which exhibit binding to and blockade of voltage-gated calcium channels are useful in reducing neuronal damage related to ischemic conditions. In the above-referenced applications, test compounds were administered at the time of or up to 1 hour following the experimentally induced occlusion which caused the ischemic event. As reported below, and according to an important feature of the invention, it has been found that there is little or no loss of protective effect of the neuroprotective compound when it is administered well after the ischemic event e.g., one hour following the period of transient occlusion. This delayed-administration protective event indicates that these compounds are effective in blocking the events leading from ischemic injury to secondary cerebral injury, since these events may occur over a period of many hours or even days after injury. Thus, the delayed administration may be effective to reduce secondary cerebral damage over a several hour period, or even a day or more, following the onset of ischemia.

The effectiveness of the composition in reducing neuronal damage related to ischemic injury has been examined in three animal systems which are widely employed as model systems for global ischemia and secondary stroke damage. The first system is the gerbil two vessel occlusion model of global ischemia produced by transient occlusion of carotid arteries of the neck. For clinical comparisons, the ischemia produced in this model has been likened to that produced by cardiac arrest, since all blood flow to

the brain is stopped for a fixed period, typically 5-10 minutes.

Although some differences in particular sequelae have been noted among species, gerbils exhibit the 5 same kind of selective regional damage from ischemia as is found in other mammals, including humans. In particular, the characteristic secondary damage observed in the hippocampal CA1 region is similar to that seen in other mammals, including humans (Kirino; 10 Yamaguchi). Neurons in this area, and especially pyramidal neurons, exhibit a delayed neuronal death over a period of up to 4 days after ischemic injury.

The second animal model utilized in experiments carried out in support of the present invention is 15 the rat four-vessel occlusion model. The experimental procedure for producing temporary occlusion produces an ischemia that mimics conditions in the human brain following cardiac arrest, including the following similarities: the ischemic 20 event is temporary, typically 5-30 minutes; it occurs in an unanesthetized state; in most rats, the ischemic event is not accompanied by generalized seizures, and animals that have seizures can be excluded from the study. In addition, the occlusion 25 procedure allows the animals to be easily monitored, maintained and analyzed (Pulsinelli).

The third animal model is the rat cerebral artery occlusion model of focal ischemia. In this model, the left middle cerebral artery is permanently 30 occluded by electrocoagulation. Twenty-four hours after the occlusion, the animals are anesthetized and areas of damage are examined by magnetic resonance imaging. Neuroprotective activity of OCT peptide MVIIA was demonstrated in this model of ischemia by

the applicants, published in PCT application WO 91/079.

A. Reduction in Anatomical Damage

5 1. Neuroprotective Activity of OCT Peptides.
Ischemia in the gerbil model system was induced in anesthetized animals by occluding the two carotid arteries for eight minutes, as detailed in Example 8. OCT peptide was administered ICV during the occlusion 10 period, or one hour following occlusion. Four to five days after occlusion and peptide treatment, the animals were examined histologically for anatomical damage in the hippocampal CA1 region, as detailed in Example 8.

15 Figures 10A and 10B are low-power micrographs of gerbil hippocampus CA1 region in animals after ischemia, and infusion of MVIIA OCT (SNX-111) (10A) or drug vehicle (10B). The arrows in the figures indicate the approximate borders of the CA1 region. 20 At higher power, cells in the drug-treated ischemic animals appear normal (Figure 11A), whereas damage is apparent in the ischemic animals receiving vehicle alone (Figure 11B). Another example of complete drug protection is seen in Figure 11C, and an example of 25 partial protection is seen in Figure 11D, where there is a small number of damaged cells.

Anatomical sections, such as those seen in Figures 10 and 11, were scored according to the criteria set out in Example 8. The extent of 30 protection from ischemic damage in animals treated with neuroprotecting OCT peptides MVIIA and OCT GIVA was reported in PCT publication WO 91/079 for peptides administered prior to or 1 hour subsequent to ischemia.

Ischemia in the rat model system was induced using the rat 4-VO method described in Example 9.

Table 5 shows protection by OCT peptides MVIIA (SNX-111) and SNX-207 against ischemic damage to the hippocampus. Both compounds showed significant neuroprotection at the doses indicated, when the compounds were administered intravenously 1 hour post-ischemia (Table 5).

10

Table 5

Effect of SNX-111 and SNX-207 on Hippocampal Damage in Rats by 4-VO

	<u>Treatment</u>	<u>Dose</u>	<u>N</u>	<u>Mean Score (SEM)</u>
	Saline	-	18	3.4 (0.5)
15	SNX-111	1 μ g	19	***1.4 (1.2)
	SNX-207	3	19	***1.4 (1.3)
	SNX-207	10	20	***1.5 (1.4)

20 ***p<.0001, compared to control unpaired student's t test

In separate studies, a series of additional OCT peptides were tested in the same animal system. OCT MVIIA (SNX-111) and OCT TVIA (SNX-185) each showed significant neuroprotection in the studies, as reported in the applicants' above-cited US patents. SNX-195, while not significantly different from control, did show a trend toward neuroprotection. In contrast, OCT SVIB (SNX-183), SNX-202, SNX-201, and OCT SVIA (SNX-157) all showed no neuroprotective activity. These studies are described in PCT publication WO 91/079.

In a second treatment method, OCT peptide was administered intravenously, as detailed in Example 9B. The degree of neuroprotection in global ischemia produced by SNX-111 administered 1 hour post-
 5 occlusion is indicated in Table 6. "NSD" in the table indicates that the value is "not statistically different" from the saline control value. In this study a dose of 15 mg/kg SNX-111 was effective to confer significant protection against cerebral damage
 10 subsequent to cerebral ischemia.

Table 6

15 Effect of intravenous administration of OCT MVIIA (SNX-111) 1 hour post-
 occlusion on hippocampal damage in rats

	Treatment mg/kg	N	Mean Score	SEM	P
20	Saline	38	3.2	.14	-
	1	12	2.9	.18	NSD
	3	10	2.9	.28	NSD
	5	9	2.4	.31	NSD
25	15	10	1.5	.28	P<.001

2. Neuroprotective Activity of OCT Peptides by Delayed Administration. Test compound was administered intravenously 6, 12, or 24 hours post-
 30 occlusion to a rat subjected to 4-VO as detailed in Example 9B. Results of a study in which saline, 1, or 5 mg/kg of OCT MVIIA was given as a bolus intravenously 6 hours post-occlusion are shown in Table 7. In contrast to administration 1 hour post-
 35 occlusion, when compound was given 6 hours post-occlusion, a significant reduction in neuronal damage was observed at the 5 mg/kg dose. Significant

reductions in neuronal damage were also observed when the same total dose drug was administered intravenously as a slow infusion over a time period of up to 25 hours.

5

Table 7

10 Effect of intravenous administration of SNX-111 6 hours post-occlusion on hippocampal damage in rats

	Treatment mg/kg	N	Mean Score	SEM	P
15	Saline	11	2.8	0.35	NSD
	0.2	11	2.4	0.34	NSD
	1	10	1.8	0.36	NSD
	5	8	0.9	0.34	p=.002*

20 *Significance determined by unpaired student's T test

25 The dose of SNX-111 required for reducing damage to the hippocampus was reduced significantly when the compound was administered 6 hours rather than 1 hour post-occlusion. Figure 15 shows a comparison of dose-response data obtained from rats subjected to 15 minutes of reversible forebrain ischemia in the four-vessel occlusion paradigm, then given varying doses of SNX-111 1 hour (triangles) or 6 hours (circles) after reperfusion. In the plot shown, damage scores from 7-21 animals are expressed as percentages of saline control values. The monophasic dose-response curves yield ED₅₀ values of 2.3 mg/kg for the animals given SNX-111 6 hours post-occlusion and 12.5 mg/kg for animals given SNX-111 1 hour post occlusion. This represents about a five-fold increase in potency

in the compound when administered 6 hours post-occlusion.

The relationship between the time of treatment and neuroprotective efficacy was further investigated by comparing damage scores in animals receiving a single intravenous bolus injection of 5 mg/kg SNX-111 at 6, 12, 24, or 48 hours after the ischemic insult. SNX-111 provided highly significant neuroprotection ($p < 0.001$) whether administered at 6, 12, or 24 hours after reperfusion (Fig. 16). Damage scores were not significantly different between control animals and those receiving SNX-111 when vehicle or peptide was administered 48 hours after ischemia. *** Figure 17 shows that SNX-159, given at a bolus dose of 10 mg/kg 6 hours post ischemia, provides neuroprotection against forebrain ischemia. Shown in the bar graph is a comparison of the effects of 3 and 10 mg/kg SNX-159 and 3.5 mg/kg SNX-111 to saline treated animals.

Although the time at which neuronal damage is observed after temporary forebrain ischemia varies among neuronal populations, maximal damage is typically manifested within 72 hours after reperfusion (Pulsinelli, Kirino). To insure that administration of SNX-111 6h post-occlusion prevented, and not simply delayed, cell loss after the ischemic insult, hippocampal damage was compared in SNX-111 treated animals after 5 or 12 days of survival. Damage scores of SNX-111 treated animals were comparably reduced at both time points (Fig. 18), suggesting that SNX-111 was not merely postponing neuronal death.

B. Functional Activity Protection

1. Hyperactivity. One common consequence of cerebral ischemia in animals is hyperactivity, which can be seen as pacing (exploratory) behavior within a few hours of occlusion, and can be observed up to 5 several days later. Hyperactivity in ischemic gerbils, was monitored as described in applicants' U.S. Patent 5,051,403, and PCT application WO 91/079, both of which are incorporated herein by reference. As describe, at three days following ischemia, SNX-10 111 treated animals showed near-normal levels of activity, indicating that the OCT peptide treatment provided protection against ischemia-induced hyperactivity.

15 2. Spontaneous Alternation. Damage to the hippocampal region of the brain is known to produce deficits in spatial learning and memory, and therefore it could be expected that ischemic damage to hippocampal cells, as documented above, might also 20 be accompanied by loss of functional activity related to short-term memory.

25 One test which has been widely applied as a measure of short-term memory in experimental animals is the Y maze. Methods of carrying out these experiments and results of neuroprotective OCT peptides were previously published in PCT application WO 91/079.

30 In these experiments, it was shown that ischemic animals in which OCT peptide treatment to significantly reduced anatomical damage, also showed statistically improved functional activity was observed, as evidenced by peptide protection against ischemia-induced hyperactivity and loss of short-term memory.

IV. Neuroprotective OCT Peptide Compounds

A. Selection of OCT Peptides

Based on a sequence homology analysis of the peptides whose full sequences are known (Figure 1), the naturally occurring neuroprotective OCT peptides were grouped into distinct groups I and II, each with internal homologies distinct to that group, as can be appreciated from Figure 14. Group I includes active OCT peptides MVIIA (SNX-111) and MVIIIB (SNX-159) which possesses a binding constant to the MVIIA site within the range of compounds with neuroprotective activity. Group II includes neuroprotective peptides GVIA (SNX-124), TVIA (SNX-185) and SNX-207. A third group includes inactive peptides SVIA (SNX-157), SVIB (SNX-183), SNX-230 and OCT peptides whose binding activities for the MVIIA site on neuronal membranes and/or activity in norepinephrine inhibition are outside the range of active compounds.

The three groups of OCT peptides are arranged in Figure 14 with their six Cys residues aligned, which places these residues at positions 1, 8, 15, 16, 20, and 28. To make this alignment, gaps were introduced at the positions shown in the three groups. Regions of sequence homology are indicated within R groups I and II by lower case letters a-f and s-v, respectively. In the analysis below, these gaps retain the assigned number shown in Figure 14, even though they represent amino acid deletions in the respective groups of active OCT peptides.

Sequence variation in the peptides, based on primary structure alone, was analyzed by adopting the following constraints:

1. The peptides in both active groups (I and II) include the Cys residues at position 1, 8, 15, 16, 20, and 28. Other Cys residues could be

substituted at the positions indicated below only if they are selectively protected during oxidation of the peptide to form the three disulfide linkages.

2. The peptides in the active groups include
5 three disulfide linkages connecting the Cys residues at positions 1 and 16, 8 and 20, and 15 and 28. According to the synthetic method described in PCT publication WO 91/079, the disulfide bridges are formed by air oxidation of the full sequence peptide
10 in the presence of DTT. The ability of the peptide to form the three desired disulfide linkages would therefore require that the peptide, prior to disulfide bridging, be able to adopt a conformation which allows the three selected linkages, with or
15 without the Cys protecting-group strategy discussed above. This constraint would thus exclude amino acid variations which prevent or otherwise hinder the formation of the three selected bridges.

Constraints 1 and 2 preserve the basic
20 conformation of the OCT peptides imposed by the three disulfide bridges.

3. Within Group I, the amino acid variations which occur at the six non-conserved residues are allowed, including peptides in which the carboxy
25 terminus is amidated or has a free acid form. That is, the first group compounds include the peptide structures having the form: Region a (SEQ ID NO: 26)-X₁-Region b (SEQ ID NO: 27)-X₂-Region c (SEQ ID NO:
28)-X₃X₄-Region d (SEQ ID NO: 29)-X₅-Region e (SEQ ID NO:
30 28)-X₆-Region f (SEQ ID NO: 30)-t, where X₁=K or S; X₂=S or H; X₃=L or T; X₄=M or S; X₅= N or a deletion; X₆=S or deletion, and t= a carboxy or amidated carboxyterminal group.

4. Within Group II, the amino acid variations
35 which occur at the eight non-conserved residues are

allowed, including peptides in which the carboxy terminus is amidated or has a free acid form. Thus, the second group compounds include the peptide structures having the form: Region s (SEQ ID NO: 27)-
5 X_1 -Region t (SEQ ID NO: 31)- $X_2X_3X_4$ -Region u (SEQ ID NO: 31)- $X_5X_6X_7$ -Region v (SEQ ID NO: 27)- X_8 -t, where $X_1=K$ or L; $X_2=X$ or R; $X_3=T$ or L; $X_4=S$ or M; $X_5=T$ or S; $X_6=K$ or R; $X_7=R$ or K; and $X_8=Y$ or R, and t= a carboxy or amidated carboxyterminal group.

10 5. Considering both active groups together, amino acid positions which are conserved in all active species are preserved. Thus, for example, the Cys residues, the 5-position glycine, the 13-position tyrosine, the 19-position serine, and the 26-position lysine are all preserved.

15 6. Considering both active groups together, there are amino acid positions which are likely to be variable within the range of active species. For example, the position 2 amino acid may be lysine or leucine, the position-3 amino acid may be glycine or serine, and the position 4 amino acid, hydroxyproline or arginine. In addition, if the two or more amino acids at a variant position are in a common substitution class, substitution within that class 20 may be favorable. Standard substitution classes are the six classes based on common side chain properties and highest frequency of substitution in homologous proteins in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix (Dayhoff).
25 These classes are Class I: Cys; Class II: Ser, Thr, Pro, 4Hyp, Ala, and Gly, representing small aliphatic side chains and OH-group side chains; Class III: Asn, Asp, Glu, and Gln, representing neutral and negatively charged side chains capable of forming 30 hydrogen bonds; Class IV: His, Arg, and Lys,
35

representing basic polar side chains; Class V: Ile, Val, and Leu, representing branched aliphatic side chains, and Met; and Class VI: Phe, Tyr, and Trp, representing aromatic side chains. In addition, each 5 group may include related amino acid analogs, such as ornithine, homoarginine, N-methyl lysine, dimethyl lysine, or trimethyl-lysine in class IV, and cyclohexylalanine or a halogenated tyrosine in Group VI. Further, the classes may include both L and D 10 stereoisomers, although L-amino acids are preferred for substitutions.

7. Considering the known inactive species, substitutions to amino acids which are present in inactive species, but not active ones, at any 15 selected residue position, are not favored to preserve activity in the active compounds. Thus, for example, although a 3-position serine is present in both active and inactive compounds, 4-position serine or threonine is present in inactive species 20 only, and either substitution is thus disfavored.

The above amino acid selection rules 6-7 are intended as a guide for allowed amino acid substitutions within neuroprotective OCT peptides. Once an amino acid substitution or modification is 25 made, the peptide is further screened for the requisite calcium channel antagonist activity, and the requisite activities for inhibition of norepinephrine release and binding to the MVIIA (SNX-111) binding site of neuronal membranes, as described 30 above.

Several of the amino acid substitutions or modifications to the OCT peptide illustrate the principles outlined above. For example, with 35 reference to Figure 2, the SNX-195 compound contains a Lys to Ala substitution at the position

corresponding to position 26 in the MVIIA structure shown in Figure 14. Since this substitution is at a conserved-sequence position, it is predicted that the neuroprotective activity would be lost or reduced.

5 As discussed above, the SNX-195 peptide shows retention of MVIIA binding activity, but reduced norepinephrine release inhibitory activity, and weak neuroprotective activity compared with the unsubstituted MVIIA OCT.

10 As another example, the SNX-201 compound contains substitutions at positions 9-12 from Ser-Arg-Leu-Met to Arg-Lys-Thr-Ser, the sequence at positions 9-12 in the inactive SVIB OCT peptide. The position-9 substitution is not favored since Arg is 15 present at this position in a non-neuroprotective compound, but not in one of the neuroprotective OCT peptides. The position-10 substitution is disfavored for the same reason. The position-11 substitution is favored, however, since the Leu to Thr substitution 20 occurs within the neuroprotective peptides. The Met to Ser substitution at position 12 is favored for the same reason. Since the peptide modification contains two disfavored substitutions, it is predicted that the neuroprotective activity would be lost or 25 reduced. As seen above, the SNX-201 peptide shows retention of MVIIA binding activity (Table 3), but reduced norepinephrine inhibitory activity (Table 2), and no neuroprotective activity at a concentration at which the unsubstituted MVIIA OCT/SNX-111 was found 30 to be active (Figure 12).

B. OCT Peptides

The invention further includes the active OCT peptides formed according to amino acid selection 35 rules 3 and 4 above, excluding the natural C-terminal

amidated OCT peptides MVIIA (SNX-111), MVIIIB (SNX-159), GVIA (SNX-124), and TVIA (SNX-185). More specifically, with reference to conserved regions a-f and s-v illustrated in Figure 14, the peptide compounds of the invention have the form: Region a (SEQ ID NO: 26)-X₁-Region b (SEQ ID NO: 27)-X₂-Region c (SEQ ID NO: 28)-X₃X₄-Region d (SEQ ID NO: 29)-X₅-Region e (SEQ ID NO: 28)-X₆-Region f (SEQ ID NO: 30)-t, where X₁=K or S; X₂=S or H; X₃=L or T; X₄=M or S; X₅=N or a deletion; X₆=S or deletion, and t= a carboxy or amidated carboxyterminal group, excluding the peptides in which X₁=K, X₂=S, X₃=L, X₄=M, X₅=deletion, and X₆=S; and X₁=S, X₂=H, X₃=T, X₄=T, X₅=N, and X₆=deletion; and Region s (SEQ ID NO: 27)-X₁-Region t (SEQ ID NO: 31)-X₂X₃X₄-Region u (SEQ ID NO: 31)-X₅X₆X₇-Region v (SEQ ID NO: 27)-X₈-t, where X₁=K or L; X₂=X or R; X₃=T or L; X₄=S or M; X₅=T or S; X₆=K or R; X₇=R or K; and X₈=Y or R, and t= a carboxy or amidated carboxyterminal group, excluding the peptides in which X₂ = X, X₃ = T, and X₄ = S.

These peptides are intended for formulation with a suitable pharmaceutical carrier, in the composition of the invention.

25

v. Selecting Neuroprotective Compounds

In vitro and in vivo studies reported in co-owned U.S. Patent No. 5,051,403 for "Method of Treating Ischemia-Related Neuronal Damage" and in PCT application WO 91/075 demonstrate a strong correlation between (a) high binding affinity to synaptosomal membranes, (b) inhibition of voltage-gated calcium ion currents and neurotransmitter release selectively in neuronal cells, and (c) ability to reduce neuronal damage in ischemia-related

injury, such as stroke. The mechanism of neural protection by high-affinity OCT peptides presumably involves inhibition of voltage-gated calcium currents in neuronal membranes, thus blocking calcium influx into neuronal cells and the consequent release of neurotransmitters from the cells. This mechanism of OCT protection is consistent with the finding that neuronal damage in ischemia-related injury is associated with elevated intracellular calcium levels (Deshpande et al.).

In the present invention, it is further demonstrated that compounds which have a high selectivity ratio of binding for the OCT MVIIA binding site (site 1), in comparison to binding to OCT MVIIC binding sites (site 2) in neuronal tissue, as determined by above-described methods, correlate well with neuroprotective activity.

In practicing the screening method of the invention, compounds are tested for their binding affinities to OCT-MVIIA and OCT-MVIIC binding sites, as described above, by their ability to displace OCT MVIIA and OCT MVIIC, respectively, from synaptosomal preparations. Binding affinities for the two sites are determined, as described in Example 5. The binding affinities are compared to produce a selectivity ratio of binding to the MVIIA binding site using the formula:

$$\text{Selectivity ratio} = \frac{K_i(\text{MVIIA})}{K_i(\text{MVIIC})}$$

30

Representative ratios are shown in Table 4. The test compound is selected as a candidate for a neuro-protective agent if (i) the compound exhibits relatively high affinity binding to the MVIIA binding

site (site 1), and (ii) the selectivity ratio of binding for the MVIIA site is at least as great as such ratios of the omega conopeptides MVIIA, MVIIIB, GVIA, GVIIA and RVIA. In the context of the present 5 invention relatively high affinity to the MVIIA site is approximated by the range of binding affinities of OCT peptides MVIIA, MVIIIB, GVIA, GVIIA and RVIA.

Test compounds selected by the binding assay may be further screened for their ability to selectively 10 inhibit N-type voltage-gated calcium currents in neuronal tissue, as detailed in co-owned U.S. Patent No. 5,051,403, incorporated herein by reference. Candidate compounds are further selected if their specific activity, expressed, for example, as IC_{50} , 15 in producing such inhibition of N-type voltage-gated calcium currents, is within the range of specific activities of an omega conotoxin MVIIA, MVIIIB, GVIA, GVIIA and RVIA.

Test compounds which are selected on the basis 20 of the above two screens may be further selected for their ability to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, as exemplified by evoked release of norepinephrine from central nervous tissue described above and in 25 Example 3. Candidate compounds are further selected if their specific activity, in producing such selective inhibition of N-channel mediated neurotransmitter release is within the range of specific activities of specific activities of an omega 30 conotoxin MVIIA, MVIIIB, GVIA, GVIIA and RVIA.

VI. Methods and Pharmaceutical Compositions for Treatment of Ischemic Neuronal Damage

As demonstrated in Section IV, administration of 35 compounds which are effective to selectively bind to

OCT site 1 binding sites and to inhibit neuronal calcium currents to animals subjected to a cerebral ischemic event results in reduction of neuronal damage. Surprisingly, and in accordance with the 5 present invention as described above, treatment with compounds of this class was shown to be effective, even when administration of the compound was delayed up to 24 hours following the onset of the ischemic event. This discovery has obvious implications and 10 usefulness in the clinical setting, where time which elapses between an ischemic attack, such as a stroke, and diagnosis and treatment is typically at least several hours.

It is anticipated that pharmaceutical 15 compositions containing compounds of the invention may be administered in any expedient formulation and route which results in delivery to the site of action, which is likely to be at or in close proximity to the ischemic region. Exemplary routes 20 of administration are intracerebral and intravenous (bolus or slow infusion); however, it is appreciated that other routes of administration, including but not limited to intranasal, intrathecal, subcutaneous, or transcutaneous administration may be used in 25 practicing the method of the invention.

The following examples illustrate, but in no way are intended to limit the present invention.

30

Example 1Synthesis of ω -Conopeptide OCT MVIIC

OCT MVIIC was synthesized on a replumbed ABI 35 model 430A peptide synthesizer, using standard t-butyloxycarbonyl (tBOC) chemistry, as described below. The synthesis was started from 0.4 mmole

methyl-benzhydralamine (MBHA) resin (0.61-0.66 equivalent NH₂/g; Advanced Chemical Technology), single coupling the first nine amino acids (except Arg and Asn, which are always double coupled as active esters formed from 1-hydroxybenzotriazole (HOBT) with dicyclohexyl carbodiimide (DCC)), and double coupling the remainder (the first couplings were in dichloromethane (DCM), the second couplings in dimethylformamide (DMFA) solution). Amino acid side chain protections were Arg(Tosyl), Asp(OBzl), Cys(4-MeBzl), Hyp(Bzl), Lys(chloro-benzyloxycarbonyl), Ser(Bzl), Thr(Bzl), Tyr(BrZ). In order to obtain 95+% overall efficiency of synthesis, the coupling steps were monitored with the ninhydrin test and were repeated to achieve 99.5%+ amino acid incorporation yield in each cycle.

The peptide was cleaved from the resin (1 g) in liquid HF (15 ml, -10°C- 0°C) containing 10% p-Cresol. The cleavage time varied between 80-95 minutes. The HF was removed with a strong stream of nitrogen, the oily residue was washed with cold ACOEt (3×20 ml, 30°C), filtered, and the peptide was extracted by washing the residue with 1×15 ml water, 3×15 ml 50% AcOH, 1×15 ml water. The combined aqueous extracts were lyophilized.

Typically 300 mg of crude lyophilized product was dissolved in 24 ml solution of guanidine hydrochloride (3 M), NH₄OAc (0.32 M) and DTT (~10 equivalents of the peptide). The mixture was stirred for 1-1.5 hours at ambient temperature, then diluted with 120 ml water and stirred while exposed to the atmosphere at +4°C. The progress of the oxidation was monitored with HPLC and completion was confirmed with the Ellman-test (at the end, the free SH content was usually less than 5% of the starting value).

Upon completion of the disulfide formation, the solution was acidified to pH -3.5 with acetic acid, concentration under vacuum to -15-20 ml, and gel-filtered on a Sephadex G-25 column (2.5 x 60 cm) eluting with 0.5 M AcOH. The pooled prepurified peptide fractions were further purified on a preparative HPLC column (Rainin Dynamax system, 4.14 x 30 cm, C-18 reversed phase packing material, 300 Å pore size, 12 µm particle size) using 0.1% TFA in water/0.1% TFA in acetonitrile gradient elution solvent system (40 ml/min pumping rate). The pure fractions were pooled, and lyophilized. The yield of purified peptide was usually 10-16% based on the loading capacity of the MBHA-resin.

Synthesis of other OCT peptides has been described in U.S. Patent No. 5,051,403, incorporated herein by reference.

Example 2

Calcium-Channel Antagonist Activity:

Inhibition of Ionic Currents

Ionic currents through calcium channels were examined in cells that were voltage-clamped by a single patch-clamp electrode. These whole-cell patch-clamp studies were performed mainly on N1E115 mouse neuroblastoma cells, although a variety of cell types, including human neuroblastoma cell line IMR-32, have been examined.

30 A. Current Measurement Methods

Most measurements were obtained using a bath saline that allowed examination of the calcium currents in the absence of other ionic currents. These solutions contained 80 mM NMDG (as a sodium replacement), 30 mM TEACl (to block potassium

currents), 10 mM BaCl₂ (as a charge-carrier through the calcium channels), and 10 mM HEPES at pH 7.3. Some solutions also contained 2 mM quinidine (to block potassium currents) and 3 μ M tetrodotoxin (to block sodium currents). Normal bath saline was (mM): 5 140 NaCl, 10 glucose, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 mM HEPES pH 7.3. Intracellular solutions contained 150 mM CsCl, 0.5 mM CaCl₂, 5 mM EGTA, 5 mM MgCl₂, 2 mM K₂ATP at pH 7.3-7.4. Bath saline and all internal 10 solutions were filtered before use.

Pipets were made from Corning 7052 glass (Garner Glass Company, Claremont, CA 91711), coated with Sylgard (Dow Corning, Midland, MI 48640) and fire-polished before use. Bubble numbers were 15 typically 5 to 6, with pipet resistances typically 2-5 M Ω ms. Corning 8161, Kimble, and other glasses were also used without noticeable effect on the calcium currents observed.

Recordings were carried out at room temperature 20 with an Axopatch 1-C amplifier (Axon Instruments, Foster City, CA 94404) and analyzed with pCLAMP software (Axon Instruments). Data were filtered at 1000 Hz for a typical sampling rate of .1 kHz; in all cases data were filtered at a frequency at most 1/5 25 of the sampling rate to avoid biasing. Data were collected on-line by the software. Analysis was performed on-screen with print-out via a Hewlett-Packard LaserJet Printer (Hewlett-Packard, Palo Alto, CA 94306).

30 The typical experiment was conducted as follows: after seal formation followed by series resistance compensation and capacitative transient cancellation, a voltage clamp protocol was performed wherein the cell potential was stepped from the holding potential

(typically -100 mV) to test potentials that ranged from -60 mV to +20 mV in 10 mV increments. The cell was held at the holding potential for 5 seconds between pulses. Protocols starting from other holding potentials usually covered the same range of test potentials.

5 B. Current Inhibition Measurement

10 Figure 3A shows calcium current traces from an N1E-115 mouse neuroblastoma cell. The figure is read from left to right in time, with downward deflections of the trace indicating positive current flowing into the cell. Currents were elicited by a voltage step from 100 mV to -10 mV. The cell was bathed in saline 15 with sodium replaced by NMDG and 10 mM Ba⁺⁺ instead of 2 mM Ca⁺⁺. Potassium currents were blocked by TEA in the bath and Cs⁺ in the pipet solution.

20 The three traces in Figure 3, labeled b-d, show decreasing calcium currents, with increasing MVIIA OCT peptide concentrations of 10 nM (b), 50 nM (c), and 200 nM (d).

25 The response of voltage-gated calcium current to increasing dosages of OCTs MVIIA and GVIA are shown in Figure 3B. The calculated IC₅₀ is approximately 10 nM for GVIA and 100 nM for MVIIA. These values indicate extremely high specificity of the peptides for their site of action.

30 Table 1 compares IC₅₀ values for GVIA, MVIIA, SVIB and SVIA OCTs. Whereas OCT GVIA and OCT MVIIA show 50% inhibition of the measured calcium current at nanomolar concentration range, IC₅₀ values for OCT SVIB and OCT SVIA were not measurable within the range of concentrations tested, and are therefore listed as having IC₅₀ values above the micromolar

concentrations indicated. OCT SVIB and OCT SVIA are considered to be inactive in this assay.

Example 3

5 Inhibition of Neurotransmitter Release

A. [³H]Norepinephrine Release from Rat Hippocampal Slices

Male Sprague-Dawley rats were lightly anesthetized with ether, decapitated, and the brains removed. The hippocampi were then dissected free of cerebral cortex and rinsed with room temperature oxygenated uptake buffer (0.1% bovine serum albumin (BSA), and in mM: NaCl, 123; KCl, 4.8; CaCl₂, 1.2; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 11; NaHCO₃, 25). Slices (200 or 400 μ M thick) were made using a McIlwain Tissue Chopper and were immediately transferred to room temperature uptake buffer. Slices were then distributed to individual wells of a 96-well plate (Dynatech) containing 0.1 ml uptake buffer per well. [³H]Norepinephrine (3 uCi/ml) diluted in uptake buffer containing 1 mM ascorbate and test compound was then added to each well. Incubation was at 37 degrees for 30 minutes in a humidified, 5% CO₂ incubator. Bathing buffer was then removed and slices washed two times for 11 minutes each with basal buffer containing appropriate test compound (basal buffer: 0.1 % BSA and, in mM: NaCl, 123, KCl, 5.0; CaCl₂, 0.4; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 11; NaHCO₃, 25). Each slice was then incubated for 15 minutes in 0.1 ml of basal buffer. This buffer was then removed for measurement and replaced by 0.1 ml stimulation buffer (0.1 % BSA in mM: NaCl, 97; KCl, 30; CaCl₂, 0.4; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 11; NaHCO₃, 25) for 15 minutes. Stimulation buffer was then removed for measurement

of radioactivity. Radioactivity remaining in each slice was determined. Data were normalized to total cpm of radioactivity per slice: total radioactivity = $S + B + \text{slice}$, where S is the amount of 5 radioactivity present in the stimulation buffer, and B is the amount of radioactivity present in the basal buffer. Stimulated release, as a percentage of total radioactivity = $100 (S/(S+B+\text{slice}))$, and basal release, as a percentage of total radioactivity = $100 (B/(S+B+\text{slice}))$. Concentration- effect graphs are 10 plotted as in Figure 5A and Figure 5B. Computer aided curve fitting was used to determine IC_{50} values from such data.

15

Example 4Synaptosomal Membrane PreparationsA. Mammalian-Brain Synaptosomes and Synaptosomal Membranes

Synaptosomes were prepared from rat whole brain 20 or hippocampal region of brain. Rats were sacrificed, and forebrains were removed and transferred to 10 ml ice-cold 0.32 M sucrose containing the following protease inhibitors (PI): 1 mM EGTA; 1 mM EDTA; 1 uM pepstatin; 2 uM leupeptin. Brains were 25 homogenized using a motor-driven Teflon-glass homogenizer (approx. 8 passes at 400 rpm). Homogenates from 4 brains were pooled and centrifuged at 900 xg for 10 minutes at 4 degrees. Supernatants were then centrifuged at 8,500 xg for 15 minutes. 30 Resulting pellets were resuspended in 10 ml each ice-cold 0.32 M sucrose plus PI with vortex mixing. The suspension was then centrifuged at 8,500 xg for 15 minutes. Pellets were resuspended in 20 ml ice-cold 0.32 M sucrose plus PI. The suspension (5 ml/tube) 35 was layered over a 4-step sucrose density gradient

(7ml each: 1.2 M sucrose, 1.0 M sucrose, 0.8 M sucrose, 0.6 M sucrose; all sucrose solutions containing PI). Gradient tubes were centrifuged in a swinging bucket rotor at 160,000 xg for 60 minutes at 4 degrees. The 1.0 M sucrose layer plus the interface between the 1.0 and 1.2 M sucrose layers were collected and diluted with ice cold deionized water plus PI to yield a final sucrose concentration of 0.32 M. The resulting suspension was centrifuged at 20,000 xg for 15 minutes. Pellets were then resuspended in 5 ml ice-cold phosphate buffered saline plus PI. The resulting rat brain synaptosomes were then aliquoted and stored in a liquid nitrogen containment system.

Prior to use in binding assays, synaptosomes were thawed and diluted with 3 volumes of ice cold deionized water plus PI. This suspension was homogenized using a PT 10-35 Polytron (setting 6) for two 10-second bursts. The homogenate was centrifuged at 40,000 xg for 20 minutes at 4 degrees. The resulting pellets were resuspended in about 5 ml of ice cold phosphate buffered saline plus PI. The resulting brain synaptosomal membrane preparation was aliquoted and stored at -80°C until use. Protein concentration of the membrane preparation was determined using Bradford reagent (BioRad), with bovine serum albumin as standard.

Example 5

30 OCT Peptide Binding to MVIIA (SNX-111) Binding Site in Synaptosomal Membranes

A. Saturation Binding Assay

MVIIA OCT was radiolabeled with ^{125}I -iodine by reaction with Iodogen™, essentially according to the method of Ahmad and Miljanich. Following the Iodogen

reaction, the peptide solution was chromatographed by HPLC through a C-8 reversed phase column and eluted with a gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in water/acetonitrile (40:60 vol/vol). The major peak of radioactivity following the underivatized MVIIA OCT was collected.

The binding constant (K_d) for [125 I]-MVIIA OCT to rat brain synaptosomal membranes was determined by a saturation binding method in which increasing quantities of [125 I] MVIIA OCT were added to aliquots of a synaptosomal membrane preparation (10 μ g membrane protein, suspended in binding buffer consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 μ M leupeptin, .035 μ g/ml aprotinin, and 0.1% bovine serum albumin (BSA), in a total volume of 0.5 ml). Binding at each concentration of labeled compound was determined in the absence and presence of 1 nM unlabeled MVIIA OCT to determine specific binding (as described in part B, below). The amount of labeled peptide specifically bound at each concentration was used to determine B_{max} , the concentration of specific binding sites on the synaptosomes, and K_d , following standard binding analysis methods (Bennett). Figure 6A shows a saturation binding curve of [125 I]MVIIA to rat synaptosomal membranes. Figure 6B shows a Scatchard transformation of the data, from which a calculated K_d of about 10 pM is determined.

30

B. Competitive Displacement Binding Assays

1. Binding of [125 I]-SNX-III (MVIIA). Rat brain synaptosomal membranes prepared as described in Example 3 were suspended in a binding buffer

consisting of 20 mM HEPES, pH 7.0, 75 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 μ M leupeptin, .035 μ g/ml aprotinin, and 0.1% bovine serum albumin (BSA). [125 I]-MVIIA (SNX-111) OCT (25-30,000 cpm, 5 approximately 1500-2000 Ci/mmol) and test compound were aliquoted into polypropylene tubes, in the absence or presence of 1 nM MVIIA (SNX-111) OCT to determine non-specific binding. The membrane suspension was diluted and aliquoted last into the test 10 tubes, such that each assay tube contained 10 μ g membrane protein and the total volume was 0.5 ml. After incubation for 1 hour at room temperature, tubes were placed in an ice bath, then filtered through GF/C filters (Whatman), which were pre-soaked 15 in 0.6% polyethyleneimine and prewashed with wash buffer (20 mM HEPES, pH 7.0, 125 mM NaCl, 0.1% BSA) using a Millipore filtration system. Just prior to filtration, each assay tube received 3 ml ice-cold wash buffer. The filtered membranes were washed with 20 two 3 ml volumes of ice-cold wash buffer, dried, and filter-bound radioactivity was measured in a Beckman gamma counter (75% counting efficiency).

Representative displacement binding curves for rat brain synaptosomal membranes are illustrated in 25 Figure 7A. IC_{50} values were computed from line fit curves generated by a 4-parameter logistic function. These values represent the concentration of test compound required to inhibit by 50% the total 30 specific binding of [125 I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes, where specific binding is defined as the difference between binding of [125 I]-MVIIA (SNX-111) OCT in the absence and presence of excess (1 nM) unlabelled MVIIA OCT. Non-specific binding is that binding of radiolabeled compound

which is measured in the presence of excess unlabeled MVIIA OCT. Such values serve as approximations of the relative affinities of a series of compounds for a specific binding site.

5 The binding constant (K_i) for each test substance was calculated using non-linear, least-squares regression analysis (Bennett & Yamamura) of competitive binding data from 2 assays performed in duplicate on separate occasions. The relationship 10 between K_i and IC_{50} (concentration at which 50% of labeled compound is displaced by test compound is expressed by the Cheng-Prusoff equation:

$$K_i = IC_{50} / (1 + [L]/K_d)$$

15

where IC_{50} is the concentration of test substance required to reduce specific binding of labeled ligand by 50%; $[L]$ is the concentration of [^{125}I]-MVIIA (SNX-111) OCT used in the experiment; and K_d is the 20 binding constant determined for binding of [^{125}I]-MVIIA (SNX-111) OCT to rat brain synaptosomal membranes in saturation binding experiments. Table 3 summarizes computed IC_{50} for various OCT peptides for 25 the MVIIA binding site of rat brain synaptosomal membranes.

2. Binding of ^{125}I -SNX-230 (MVIIIC). Rat brain synaptosomal membranes were prepared as 30 described above. OCT SVIB/SNX-230 was radiolabeled by iodination with ^{125}I -iodine by the Iodogen reaction, by standard procedures. Displacement binding of radiolabeled MVIIIC on rat brain synaptosomal membranes was carried out as described 35 for SNX-111, above. Results are shown in Figure 7B.

Example 6Identification of OCT MVIIA (SNX-111) and
OCTMVIIIC/SVIB Binding Proteins

Conopeptide receptor polypeptides in rat brain
5 or hippocampal synaptic membranes were chemically
cross linked to radio-iodinated SNX-111, SNX-183, or
SNX-230 with the water-soluble carbodiimide
(1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide)
procedure. The radiolabeled peptides were separated
10 by SDS-PAGE and visualized by autoradiography. For
each sample analyzed, 50 μ l of membrane protein in
50 μ l binding buffer (see above) was combined with
400 μ l binding buffer and 50 μ l of radioiodinated
peptide (0.1nM for SNX-111, and for SNX-230, and 1nM
15 for SNX-183). To corresponding control samples was
added 100 to 1000 fold excess of non-radioactive
peptide for simple determination of specific labeling
or, for concentration-dependent displacement analy-
sis, the appropriate serial dilutions of
20 non-radioactive peptide. The mixtures were incubated
at room temperature for 40 minutes. The tubes were
cooled on ice and 10 μ l of freshly prepared 25mM EDC
(in 25mM PIPES buffer, pH 6.1) and 10 μ l of freshly
prepared N-hydroxysulfosuccinimide (NHS; in 25mM
25 PIPES buffer, pH 6.1) was added to each sample.
(Corresponding control samples received no EDC or
NHS). The reaction was allowed to proceed for 10
minutes and then quenched by addition of 10 μ l 1M
NH₄Ac. The mixture was immediately pelleted for 15
30 min at 12,000xg in a microfuge, the supernatant
discarded, and washed by 2 cycles of resuspension and
pelleting with 1ml 25mM HEPES, pH 7.5. The final
pellets were dissolved in 20 μ l fresh sample buffer
and kept at room temperature for 30 min without
35 heating. Samples were run on a 4-15% gradient

SDS-polyacrylamide gel. Gels were fix, stained with Coomassie blue, dried, and exposed to autoradiographic film by standard methods. For analysis of the concentration-dependent displacement of cross-linking in particular labeled polypeptide bands, two methods were used: 1) excision of the bands and direct counting of radioactivity in a gamma counter, or 2) densitometric analysis of the bands on the autoradiogram using a computer-aided imaging system and ^{125}I microscales (Amersham) as standards for determining film response curves.

Autoradiographs showing binding to separated proteins of $[^{125}\text{I}]$ MVIIA/SNX-111 are shown in Figure 8A and Figure 8C. Binding of $[^{125}\text{I}]$ SVIB/SNX-183 and of $[^{125}\text{I}]$ MVIIIC/SNX-230 are shown in Figures 8B and 8D, respectively.

Example 7

Localization of OCT Binding Proteins

20 A. Receptor Autoradiography

Whole brain was rapidly removed from male Sprague Dawley rats (250-350 mg.) and frozen in isopentane precooled on dry ice. The frozen brain was either used immediately or stored at -80°C and then used within three days. Coronal sections ($20\mu\text{M}$) were obtained at -15 to -20°C with a crystal microtome and thaw transferred to gelatin coated glass microscope slides. The sections were stored at -80°C and usually used within four weeks. The frozen tissue sections were allowed to dry at room temperature and then incubated with $200-250\mu\text{l}$ binding buffer (NaCl (75mM), EGTA (0.1mM), EDTA (0.1mM), leupeptin (2 μM), aprotinin (0.5 unit/ml), bovine serum albumin (1.5%w/v), polylysine (MW=1000-4000, 1 μM), and HEPES/NaOH (20mM, pH 7.5)) plus peptide for

40-45 min in a humidified box at room temperature. The concentrations of radio-iodinated peptide and corresponding non-radioactive peptide for determination of specific binding were as follows:

5 0.1-0.15nM [¹²⁵I]-SNX-111 and 25nM SNX-111; 0.3 to 0.5nM [¹²⁵I]-SNX-183 and 100nM SNX-183. After the incubation period, the solution was poured off the slides and unbound peptide was then removed by serially passing the slides through four dishes (4

10 min. incubation each) of washing buffer (HEPES (50mM), NaCl (170mM), Triton X-100 (0.05%) and bovine serum albumin (0.1%)). After the final wash, the slides were dipped quickly five times in water and dried under a stream of air at room temperature.

15 Dried slides were exposed to XAR-2 X-ray film, and the film developed. The developed images were examined either directly over a light box, by inspection of enlarged photographic prints, or by a computer assisted image analyzer. The assignment of

20 binding to specific neuro-anatomical sites was made using an anatomical atlas of the rat brain (Paxinos).

Example 8

Reduction in Anatomical Damage:

25 Global Ischemia Model 1

Global ischemic damage was examined in the gerbil model, according to standard procedures (Kirino). Male mongolian gerbils (*Meriones unguiculatus*, Tumblebrook Farm, West Brookfield, MA)

30 weighing 50-80 g were anesthetized in a small chamber with 4% halothane carried by 70% nitrous oxide (0.44 L/min) and 30% oxygen (0.19 L/min). They were then maintained throughout surgery with 2% halothane by placing their noses through a hole in a

35 rubber dam on a gas delivery tube. Using aseptic

techniques, both common carotid arteries were exposed, dissected free of surrounding tissue, and occluded with microvascular clamps approximately 3 to 4 mm above the clavicle. The occlusions were 5 maintained for 8 minutes, timed while both arteries were occluded. There was generally a period of approximately 1 minute between clamping of each of the two arteries, and approximately 4 seconds between unclamping them. After the clamps were removed, the 10 skin was sutured shut and anesthesia discontinued.

During or after the occlusion, an intracerebroventricular (ICV) injection aimed at the lateral ventricle was made. To accomplish this, a 10 microliter Hamilton syringe with a 27 gauge needle 15 was filled with injectate by backloading to assure the absence of air in the system. A stiff plastic sleeve was slipped onto the needle so that 3.5 mm of the needle protruded past the sleeve. The skull around the bregma was exposed, a distance of 1.1 mm 20 left of the midline was measured with a compass, and a distance of 0.4 mm posterior to bregma was approximated by eye. The needle tip was held perpendicular to the skull and inserted through it at that point by applying gentle pressure while 25 twisting. It was advanced until the sleeve abutted the skull, and 5 microliters of injectate was infused over a period of approximately 3 sec. The skin was then sutured shut. Occluded animals received either drug or its vehicle. Injected, unoccluded controls 30 were anesthetized, and received the ICV injection only.

Four to five days after the initial occlusion, animals were anesthetized with CO₂. The chest cavity was opened and the animal was perfused through the 35 heart with approximately 3 milliliters of phosphate-

buffered saline (PBS; 0.10 M sodium phosphate; 0.15 M sodium chloride) containing heparin (10 Units/ml), followed by approximately 10 ml of Zamboni's fix (15% (vol/vol) picric acid, 4% (wt/vol) paraformaldehyde, 5 in 0.1 M phosphate buffer pH 7.4) or 10% phosphate buffered formalin. Brains were removed and left immersed in the same fixative for several hours.

Brains were blocked just posterior to the optic chiasm and posterior to the mammillary bodies. They 10 were then placed in 10% (wt/vol) sucrose in PBS overnight at 4 degrees. The block containing the hippocampus was frozen with liquid Freon onto a cryostat chuck using Tissue-Tek™ O.C.T. embedding medium for frozen tissue specimens (Miles Inc., 15 Elkhart, IA). Sections 10 microns in thickness were cut. Series of 5 sections were collected, with each series approximately 100 microns apart, until the relevant part of the hippocampus was obtained (40-50 sections per brain). At least 8 sections per brain 20 were stained with hematoxylin and eosin, substantially according to reported procedures.

Coverslips were then placed over the sections, 25 using Permount™ as an adhesive. Figures 10A and 10B are low-power micrographs of gerbil hippocampus (CA1 region) in animals after ischemia, after infusion of MVIIA OCT (10A) or after drug vehicle (10B). The arrows in the figures indicate the approximate borders of the CA1 region of the hippocampus. At higher power, cells in the drug-treated ischemic 30 animals appear normal (Figure 11A), whereas damage is apparent in the ischemic animals receiving vehicle alone (Figure 11B). Another example of complete drug protection is seen in Figure 11C, and an example of partial protection is seen in Figure 11D, where there 35 are a small number of damaged cells.

Sections, such as those seen in Figures 10 and 11, were viewed and scored by an investigator having no knowledge of the treatment of any particular sample. Ischemic damage was scored in the CA1 region of the hippocampus. Damage was generally seen as pink (eosinophilic) cytoplasm and shrunken, dark blue nuclei. Scoring was as described below:

	<u>Score</u>	<u>Observation</u>
10	0	No damaged cells were apparent.
15	1.	Less than 25% damaged cells in a CA1 field, or damage was restricted only to the extreme edges of the CA1 region.
20	2.	Approximately 50% damaged cells in a CA1 field, or damage to less than half the length of CA1, but more than to only the extreme edges.
25	3.	Damaged cells outnumber normal cells to a maximum of 75%, with damage extending throughout most of CA1.
30	4.	Complete damage to CA1, with fewer than 25% normal cells surviving.

Example 9

Reduction in Anatomical Damage:

35 Global Ischemia Model 2

Global ischemic damage was examined in the rat brain model, employing the four-vessel occlusion method of Pulsinelli and Brierly (Pulsinelli) for introducing temporary global ischemia in rats.

40 Although the two carotid arteries supply blood to the forebrain, their occlusion alone has only moderate effects on forebrain blood flow because the posterior

communicating arteries allow blood to be shunted from the brainstem blood supply, which is fed by the two vertebral arteries. Therefore, in order to effect severe forebrain ischemia, all four vessels must be 5 occluded. The procedure used allows ischemia to be produced in conscious animals, by closing surgically implanted clamps, and therefore avoid possible interactions with drug treatment. The procedure was modified to allow carotid occlusion without the need 10 for reopening a skin wound in conscious animals.

Surgery was performed to permanently occlude both vertebral arteries and to implant an arterial clasp to allow temporary occlusion of the carotid arteries at a later time. Under sodium pentobarbital 15 anesthesia (60-65 mg/kg, intraperitoneally, i.p.) male Fisher 344 rats were placed in a stereotaxic holder and the first cervical vertebra was exposed with the aid of a dissecting microscope. The vertebral arteries were occluded at the first cervical 20 artery, through the alar foramina, with a thermocautery device and the skin closed with wound clips. The animal was placed on its back and the carotid arteries were carefully dissected free of the surrounding nerves and vessels under the microscope. 25 The loose end of the Silastic loop of the clasp was passed behind the artery and put through the open side of the clasp and secured as for the other end. This was then repeated for the other carotid. The 30 clasps were tied into the skin with 3-0 suture as the skin was closed so as to externalize the ends of the loop.

Ischemia was produced 2 days after surgery. To occlude the carotid arteries, the animal was held by lightly pinching the skin at the back of the neck and 35 the ends of each loop were pulled out and secured

with a bulldog clamp. At the end of the 15 min. occlusion, the clamps were removed to allow reperfusion. An effective occlusion causes the animal to lose its righting response within about 1 min. of occlusion. If the animal did not lose the righting response or if it regained it during occlusion, the loops were pulled tighter to assure complete carotid occlusion. Animals that did not lose their righting response were eliminated from the study, because this suggested that there was still significant cerebral blood flow. This observation was confirmed by neuropathological analysis showing damage to be less in animals retaining their righting reflex than in animals that do lose their righting response. Some animals righted themselves once or twice during the occlusion but immediately lost the righting response again, and were not eliminated from the study. Any animal that righted itself and remained up was eliminated.

20

A. Intracerebroventricular Administration of OCT Peptide

Rats receiving intracerebroventricular (ICV) compound were anesthetized using halothane immediately following reperfusion, and compound contained in 5 μ L saline or saline alone was injected into the lateral ventricle as for gerbils. The coordinates of the injection were 1.2 mm left of midline and 0.5 mm posterior to bregma, at a depth of 3-4 mm. Rectal temperature was monitored from just before occlusion, and for 4-6 hours post occlusion. Rats were maintained normothermic (rectal temperature at about 37 degrees) for 4-6 hours following occlusion, by means of a heating apparatus. The degree of

neuroprotection was assessed as in Example 8 and is shown in Tables 7 and 8.

B. Intravenous Administration of OCT Peptide

5 For intravenous (IV) administration of compound in conjunction with the rat 4-VO model of cerebral ischemia, rats were subjected to surgery and subsequent occlusion as described above. For administration of compound, rats were placed into
10 Rodent Restraint Cones (Harvard Bioscience). Reversible tourniquets were applied to tail veins, and OCT compound was injected in a total volume of 0.25 ml, at the times and doses indicated in Tables 8-11. As in the case of ICV administration, rats
15 were maintained normothermic (rectal temperature at about 37 degrees) for 4-6 hours following occlusion, by means of heating apparatus. The degree of neuroprotection was assessed as in Example 8.

20 C. Neuropathology

Neuropathological analysis of rat brains was carried out essentially identically to that described for gerbil brains in Example 7. Rats were killed with carbon dioxide five days after occlusion and
25 perfused through the heart with normal saline, then with 10% buffered formalin. Brains were removed and immersed in the formalin fixative for 4-6 hours, then placed into 15% sucrose/PBS overnight. Fifteen micron sections through the dorsal hippocampus were
30 collected at approximately 300 micron intervals, stained with hematoxylin and eosin, and the sections examined microscopically for damage to the CA1 subfield of the hippocampus, as described in Example 8.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the

5 invention.

IT IS CLAIMED:

1. A method for reducing neuronal damage related to an ischemic condition in a mammalian subject, comprising administering to the subject, at a time 4-24 hours following the onset of the ischemic condition, a pharmaceutically acceptable amount of a compound effective to selectively bind to an OCT MVIIA binding site in neuronal tissue, as evidenced by a selectivity ratio of binding of the compound for the MVIIA site, determined by its binding affinity for said MVIIA site and its binding affinity to an OCT MVIIIC site, which is at least 100.

15 2. The method of claim 1, wherein the compound has a selectivity ratio of binding compound for the MVIIA site which is at least 500.

20 3. The method of claim 1, wherein the compound has a selectivity ratio of binding for the MVIIA site which is at least as great as that of an omega conotoxin selected from the group consisting of MVIIA, MVIIIB, GVIA, GVIIA and RVIA omega conotoxins.

25 4. The method of claim 1, wherein the compound is further characterized by a binding affinity at the OCT MVIIA site which is at least as great as that of an omega conotoxin selected from the group consisting of MVIIA, MVIIIB, GVIA, GVIIA and RVIA omega conotoxins.

30 5. The method of claim 1, wherein the compound is further effective to selectively inhibit N-type voltage-gated calcium currents in neuronal tissue, as evidenced by a specific activity, in producing such

inhibition of N-type voltage-gated calcium currents, which is at least as great as that of an omega conotoxin selected from said group of omega conotoxins.

5

6. The method of claim 1, wherein the compound is further effective to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, as evidenced by a specific activity, in producing 10 such inhibition of neurotransmitter release, which is at least as great as that of an omega conotoxin selected from said group of omega conotoxins.

7. The method of claim 1, wherein the compound 15 is an omega conotoxin.

8. The method of claim 7, wherein the omega conotoxin is selected from the group consisting of OCT MVIIA, OCT MVIIB, OCT GVIA, OCT GVIIA, OCT RVIA, 20 and SNX-207.

9. A method of screening compounds for use in reducing ischemia-related neuronal damage, such as produced by stroke, in a human, comprising 25 measuring binding affinities of the compounds to be screened to OCT MVIIA and OCT MVIIC binding sites in neuronal tissue,

determining from said binding affinities a selectivity ratio of binding for the MVIIA binding 30 site,

selecting a compound if its selectivity ratio of binding for the MVIIA site is at least 100.

10. The method of claim 9, wherein the compound 35 is selected if its selectivity ratio is at least 500.

11. The method of claim 9, wherein the compound is selected if its selectivity ratio of binding for the MVIIA site is at least as great as that of an omega conotoxin selected from the group consisting of 5 MVIIA, MVIIB, GVIA, GVIIA and RVIA omega conotoxins.

12. The method of claim 9, which further includes measuring the ability of the compound to selectively inhibit N-type voltage-gated calcium 10 currents in neuronal tissue, and said selecting further includes selecting the compound if its specific activity, in producing such inhibition of N-type voltage-gated calcium currents, is at least as great as that of an omega conotoxin selected from 15 said group of omega conotoxins.

13. The method of claim 9, which further includes measuring the ability of the compound to selectively inhibit N-channel mediated neurotransmitter release in neuronal tissue, and said selecting further includes selecting the compound if its specific activity, in producing such inhibition of neurotransmitter release, is at least as great as that of an omega conotoxin selected from said group of omega 25 conotoxins.

14. The method of claim 9, wherein the compound is an omega conotoxin.

30 15. An omega conotoxin peptide having the form:
SEQ ID NO: 27-X₁-SEQ ID NO: 31-X₂X₃X₄-SEQ ID NO: 32-X₅X₆X₇-SEQ ID NO: 27-X₈-t, where X₁=K or L; X₂=X or R; X₃=T or L; X₄=S or M; X₅=T or S; X₆=K or R; X₇=R or K; and X₈=Y or R, and t= a carboxy or amidated

carboxyterminal group, excluding the peptides in which $X_2=X$, $X_3=T$, and $X_4=S$.

16. A peptide having the structure SEQ ID NO:
5 20, wherein t = a carboxy or amidated carboxyterminal group.

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1 5 10 15 20 25 30

MVIIIA/
SNX- 111
C K G K G A K C S S R L M Y D C C T G S C - R - S G K - C

MVIIIB/
SNX- 159
C K G K G A S S C H R T S Y D C C T G S C N R - - G K - C

GVIA/
SNX- 124
C K S X G S S C S X T S Y N C C R - S C N X Y T - K R C - - Y

GVIIB/
SNX- 178
C K S X G T X C S R G M R D C C T - S C L L Y S N K - C R R Y

RVIA/
SNX- 182
C K P X G S X C R V S S Y N C C S - S C K S Y - N K K C G

MVIID/
SNX- 238
C Q G R G A S C R K T M Y N C C S G S C N R G R C

Fig. 1

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1 5 10 15 20 25 30

SVIA/
SNX-
157

C R S S G S X C G V T S I - C C - G R C - - Y R G K - C T

TVIA/
SNX-
185

C L S X G S S C S X T S Y N C C R - S C N X Y S - R K C R

SVIB/
SNX-
183

C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C

MVIIIC/
SNX-
230

C K G K G A P C R K T M Y D C C S G S C G R - R G K - C

SNX-
231

C K G K G A X C R K T M Y D C C S G S C G R - R G K - C

Fig. 1 (con't)

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	1	5	10	15	20	25
MVIIA (SNX-111)	C	K	G	A	K	C
	<u>S</u>	<u>R</u>	<u>L</u>	<u>M</u>	<u>Y</u>	<u>D</u>
	<u>C</u>	<u>T</u>	<u>G</u>	<u>S</u>	<u>C</u>	<u>R</u>
	<u>S</u>	<u>G</u>	<u>K</u>	<u>S</u>	<u>G</u>	<u>K</u>
						<u>C-NH₂</u>
SNX-190	-	-	A	-	-	-
SNX-191	-	A	-	-	-	NH ₂
SNX-193	-	-	-	-	-	G-OH
SNX-194	-	-	-	-	Nle-	-
SNX-195	-	-	-	-	-	A
SNX-196	N	-	-	-	-	NH ₂
SNX-197	N	9	-	-	-	G-OH
SNX-198	-	-	-	-	-	NH ₂
SNX-200	-	-	-	A	-	-
SNX-201	-	-	-	-	R	NH ₂
MVIIIC	-	-	-	P	-	R
SNX-260	-	-	-	P	-	R
					-	NH ₂

Fig. 2

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Fig. 2 (cont.)

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Fig. 3A

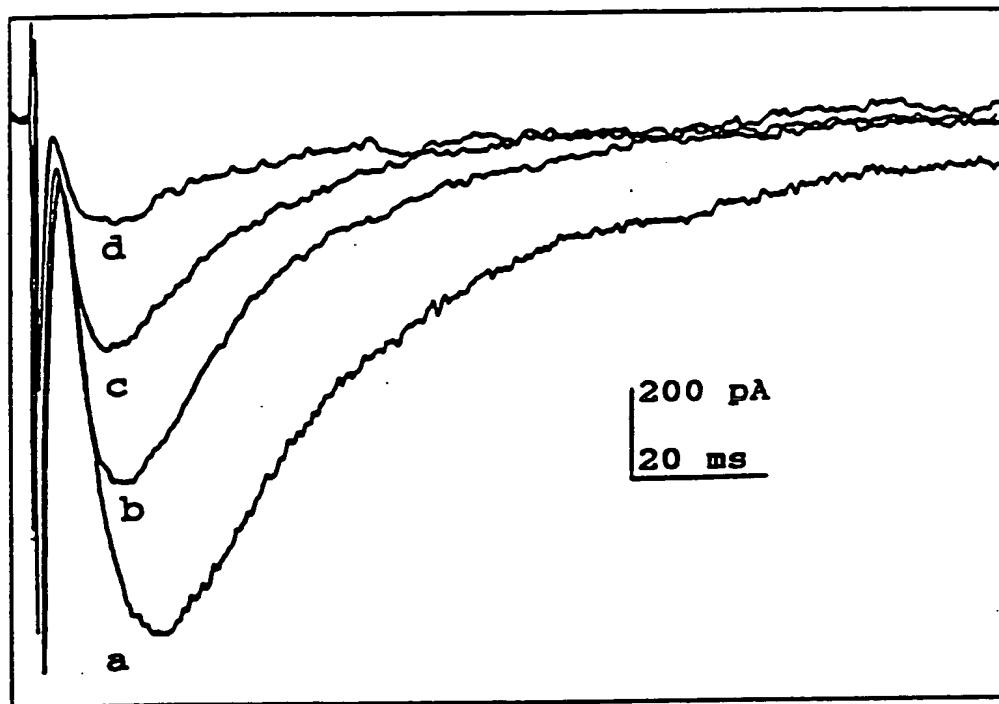
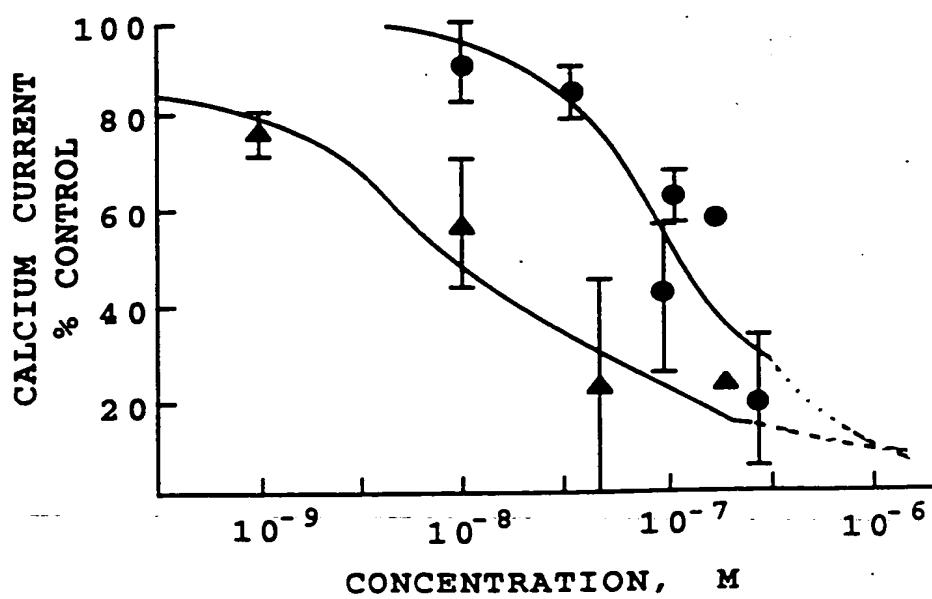


Fig. 3B



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Fig. 4A

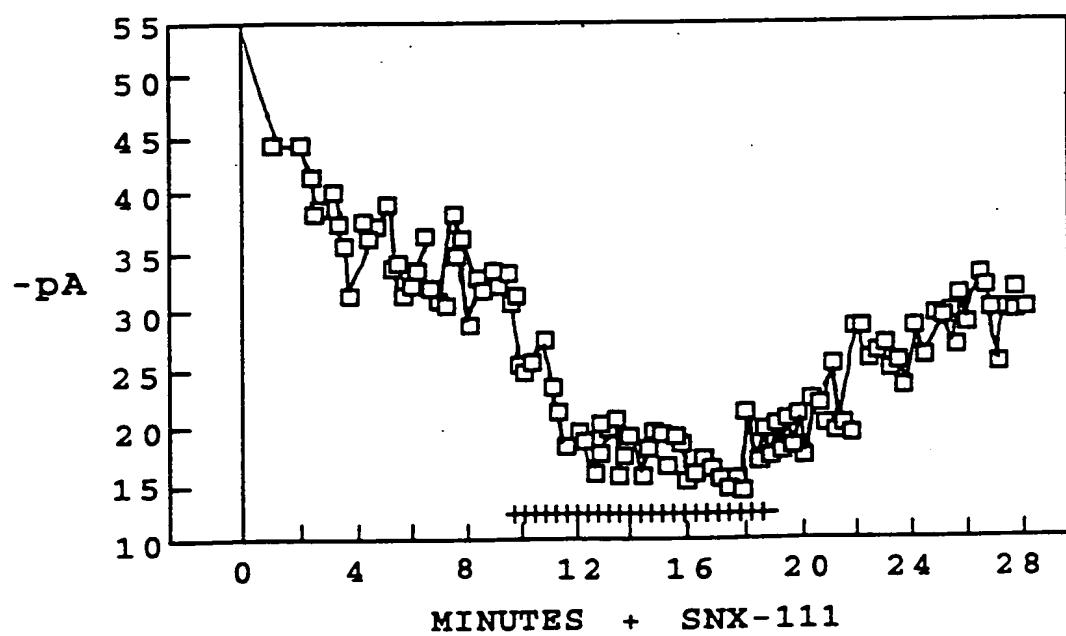
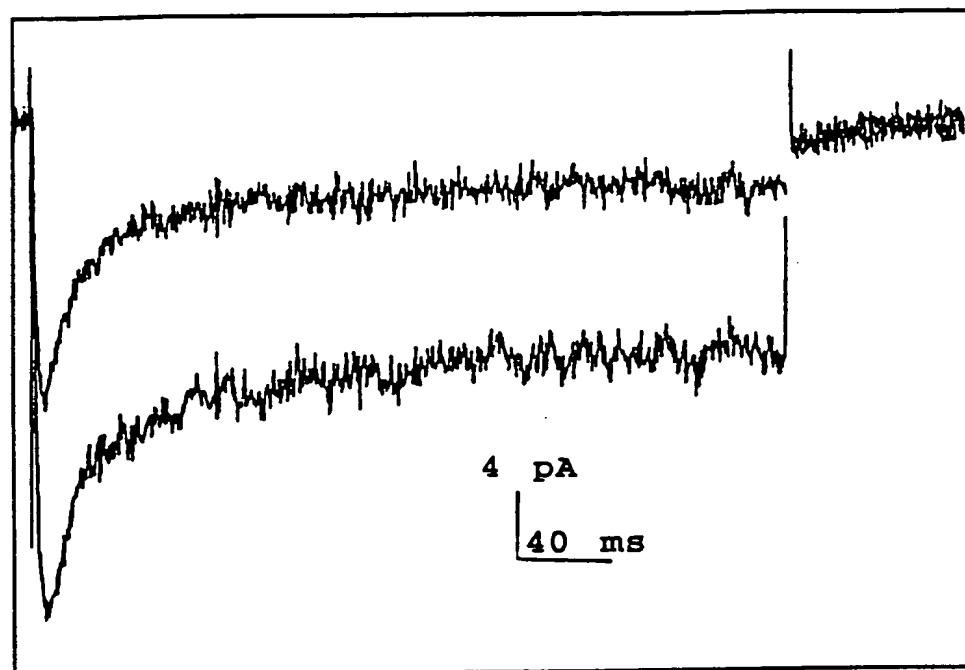


Fig. 4B

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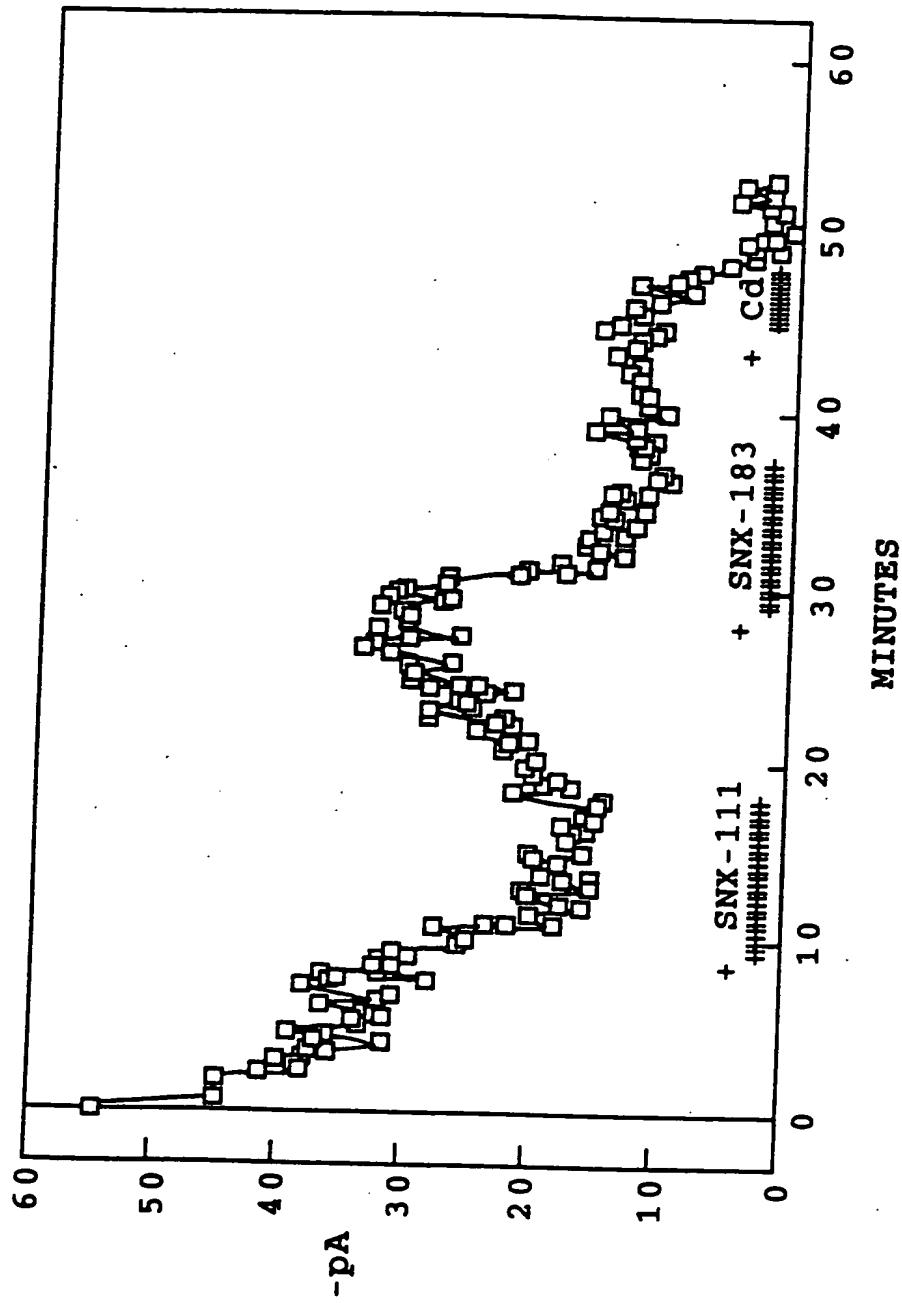


Fig. 4C

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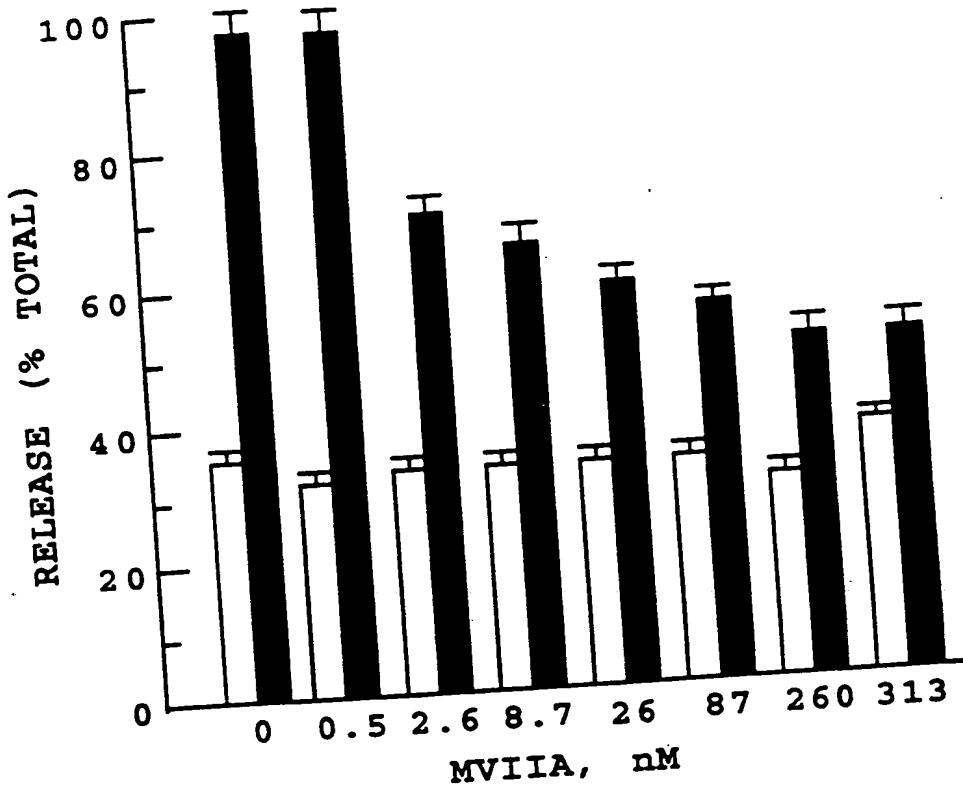


Fig. 5A

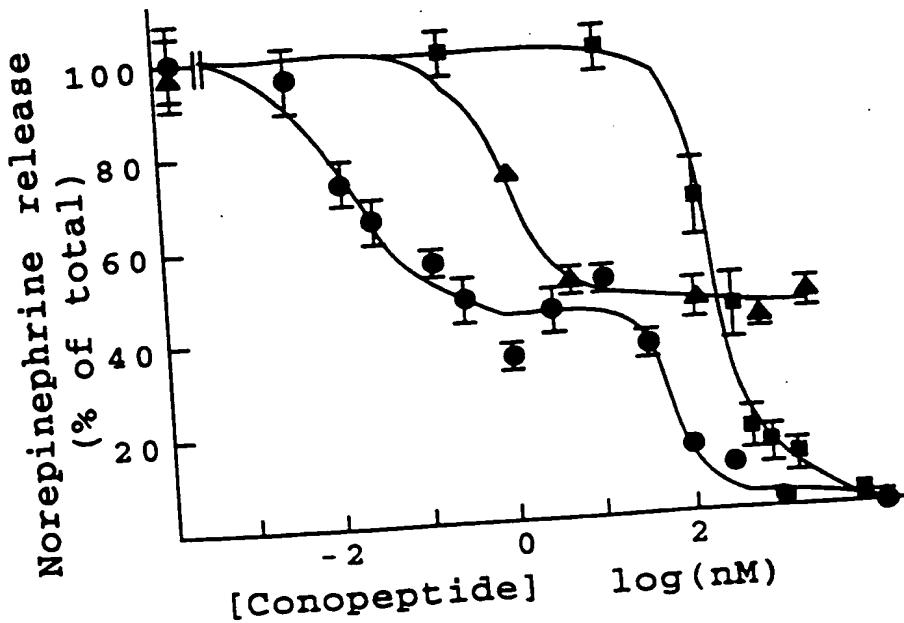


Fig. 5B

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Fig. 6A

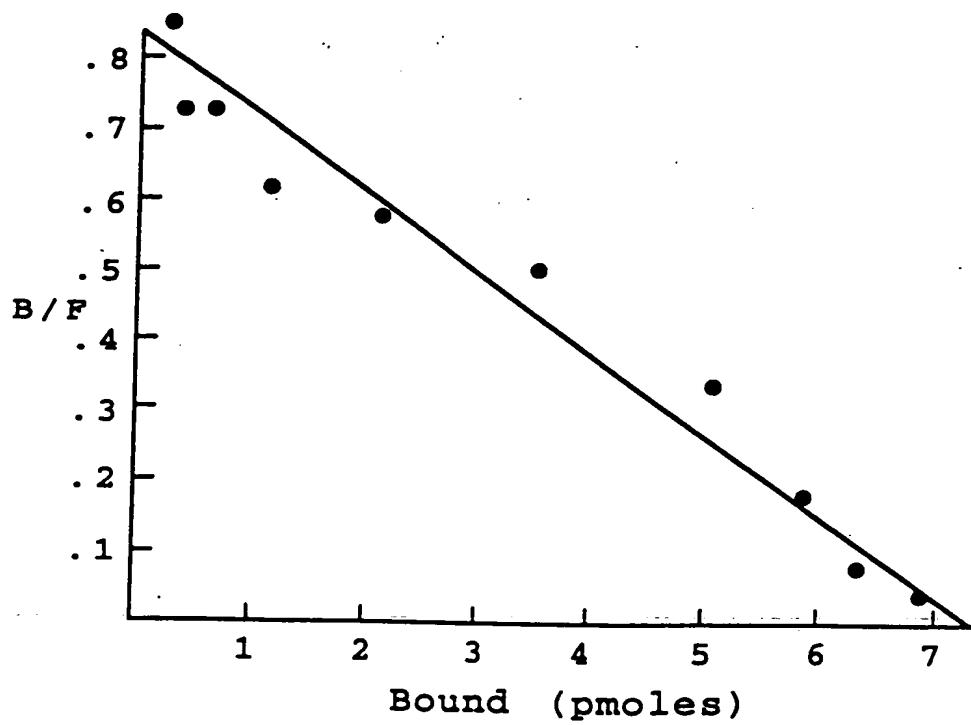
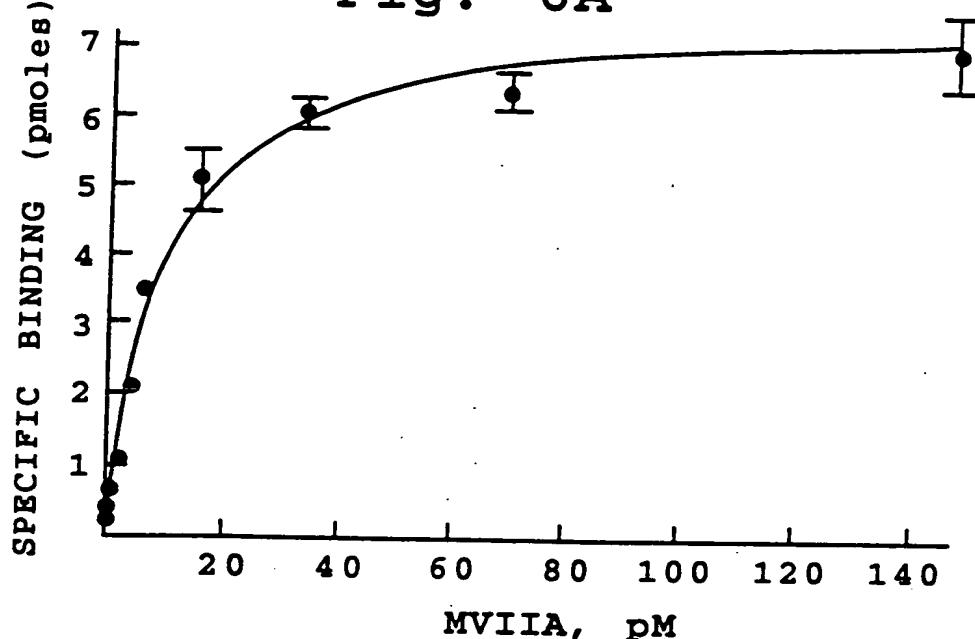


Fig. 6B

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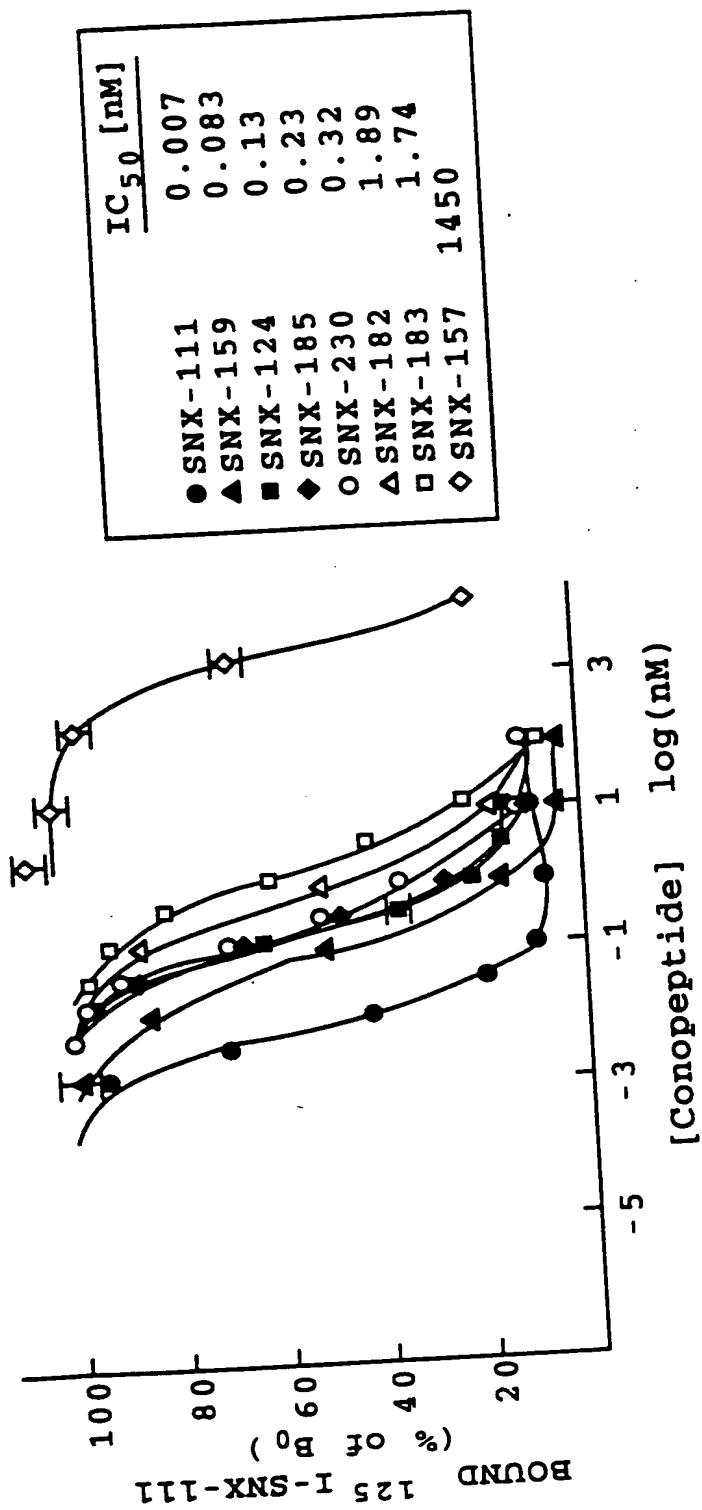


Fig. 7A

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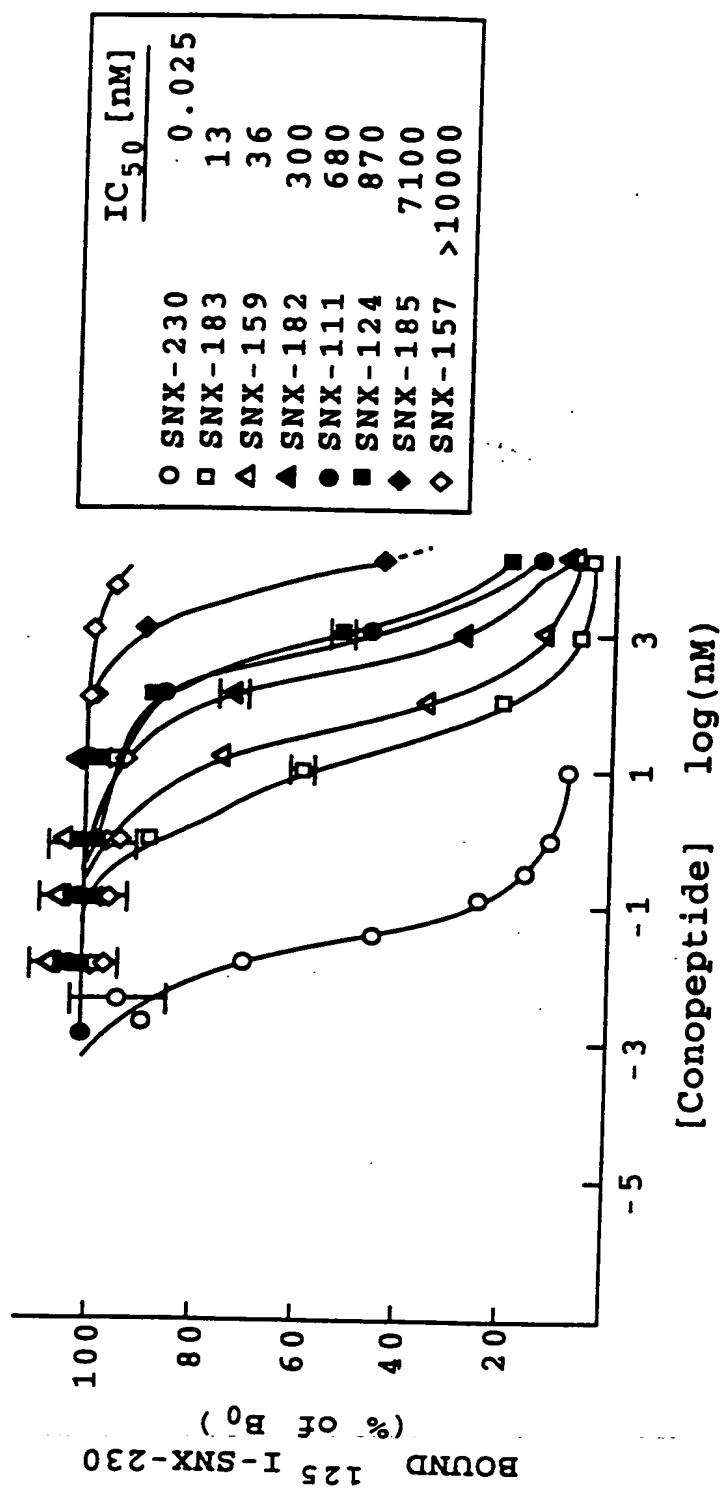


Fig. 7B

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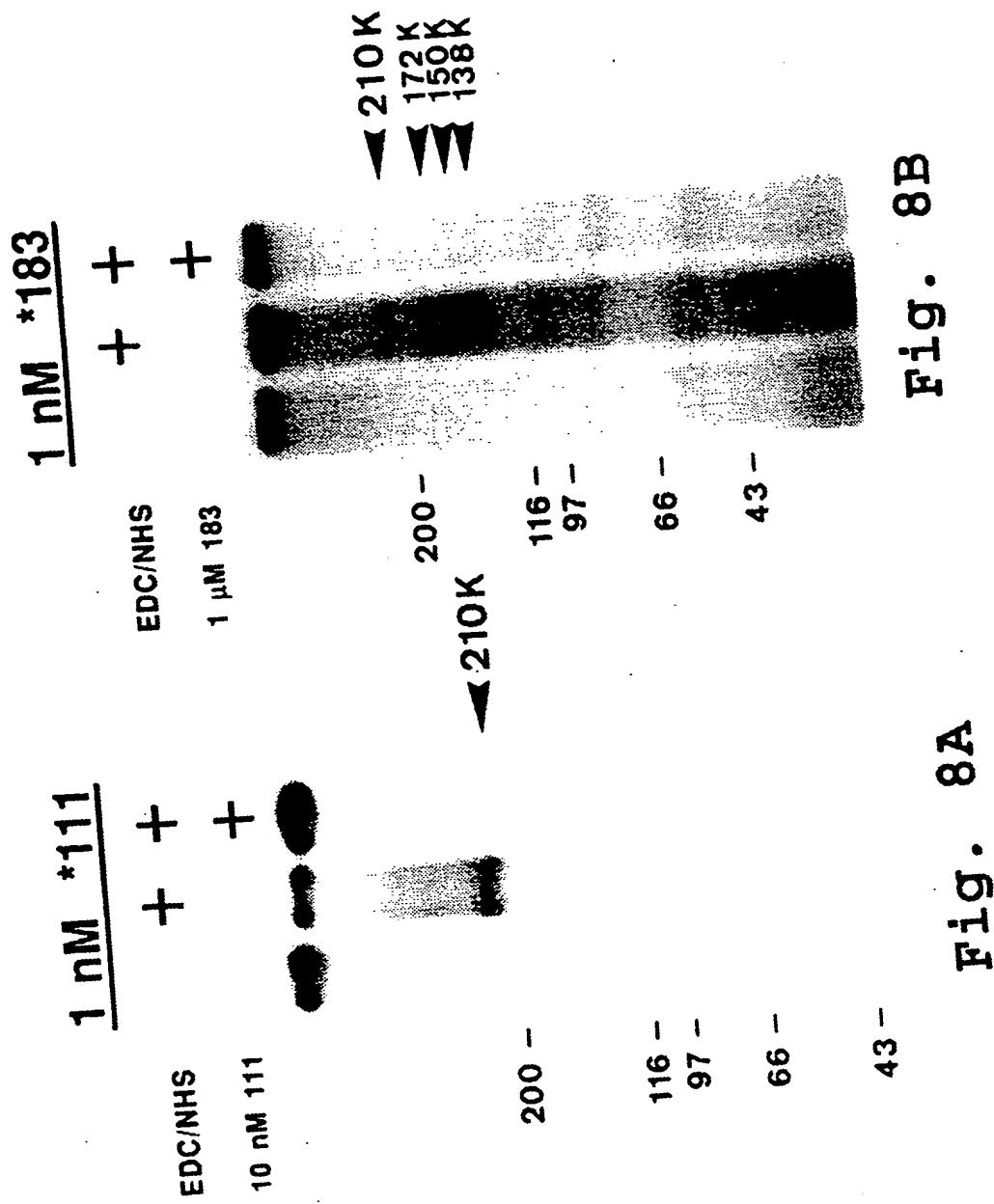


Fig. 8B

Fig. 8A

SUBSTITUTE SHEET

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125I-SNX-111

125I-SNX-230

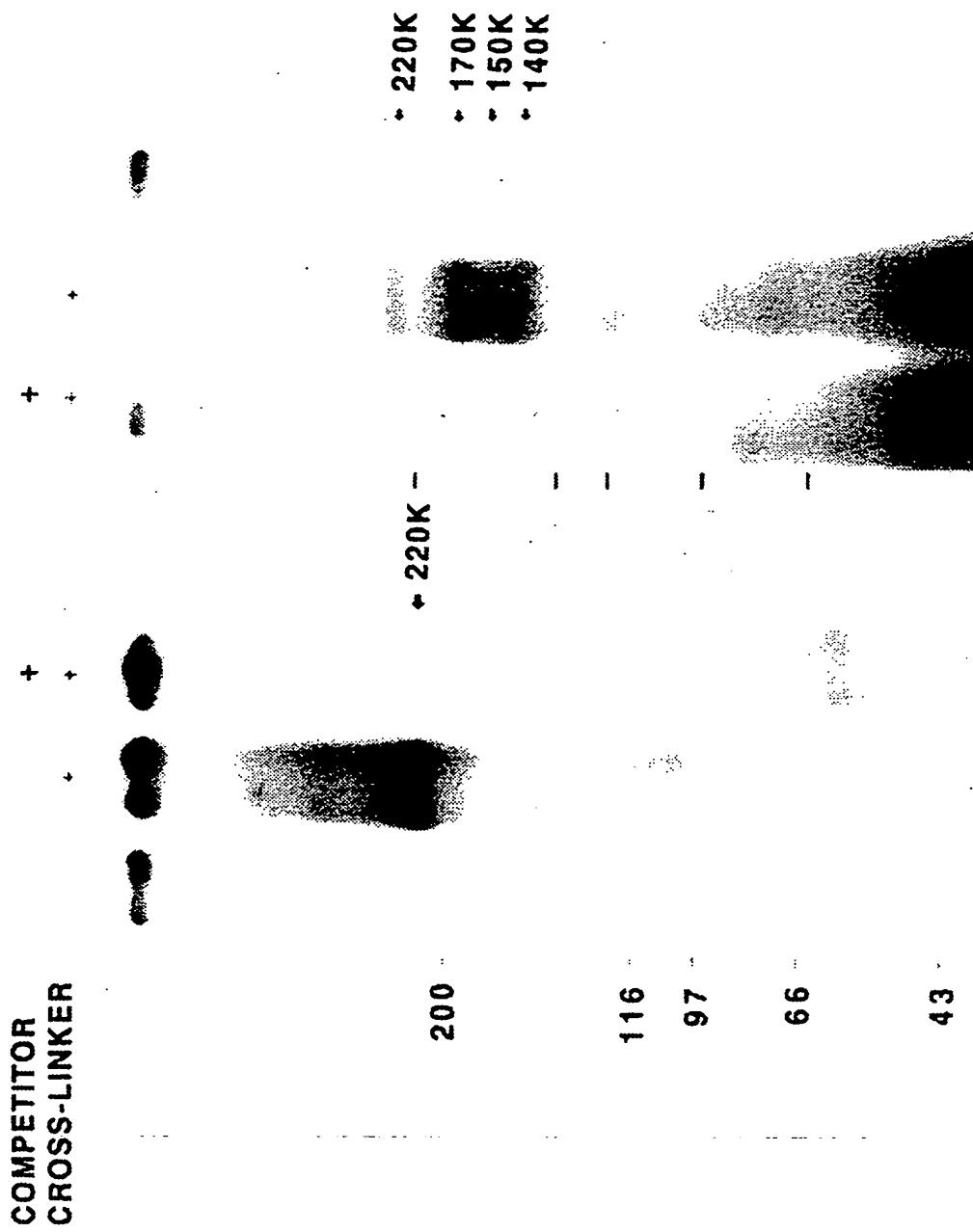


Fig. 8C Fig. 8D

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Fig. 9A

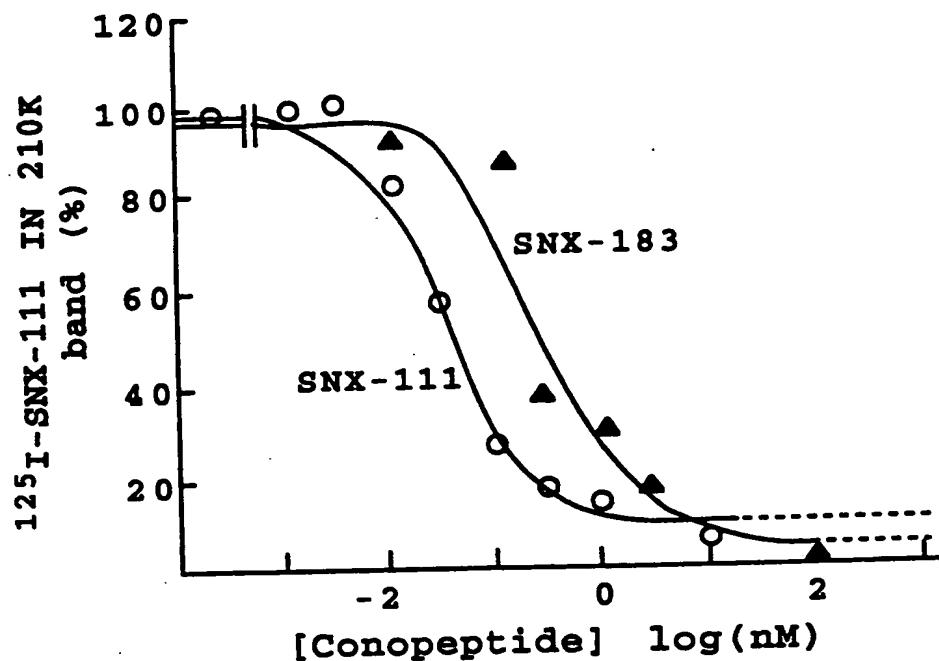
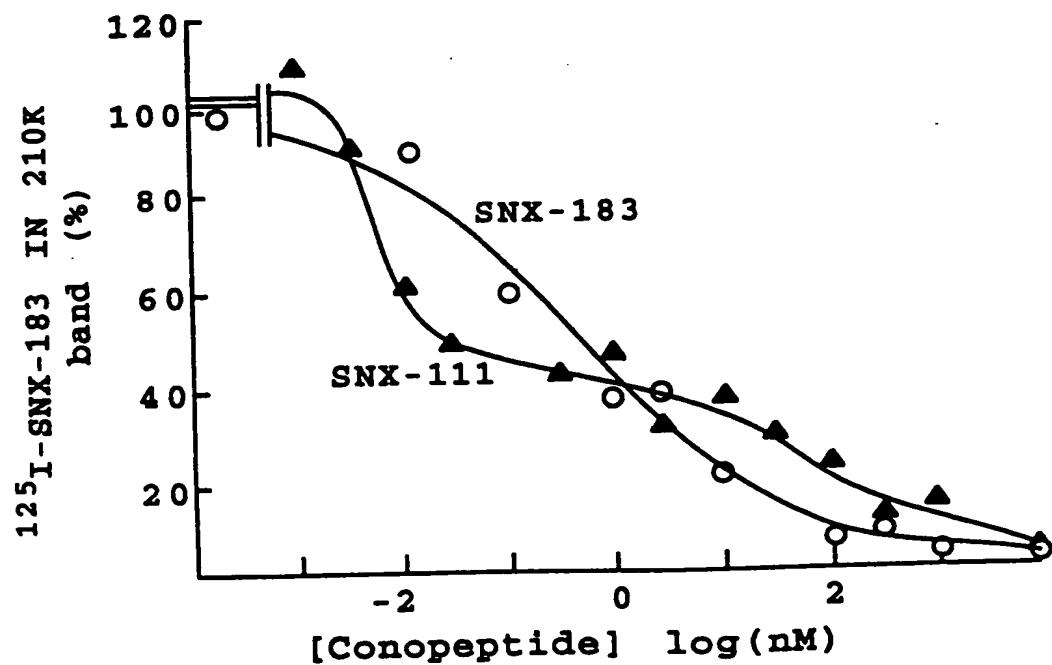


Fig. 9B



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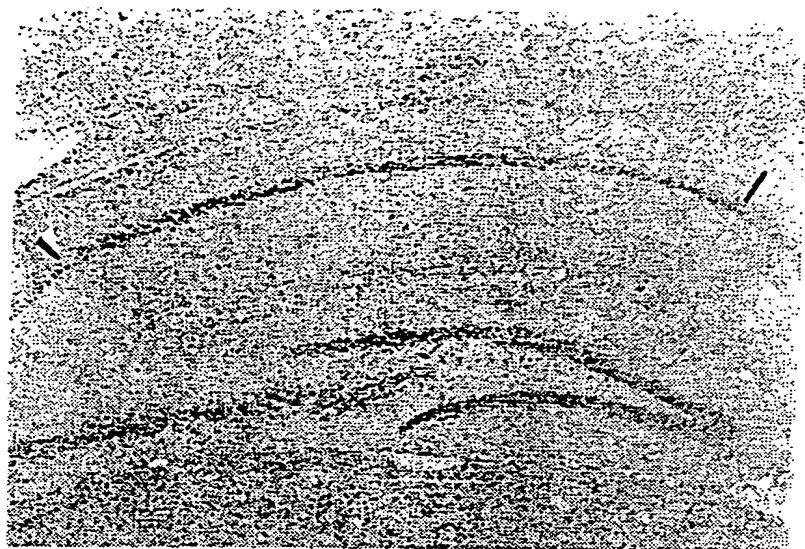


Fig. 10A

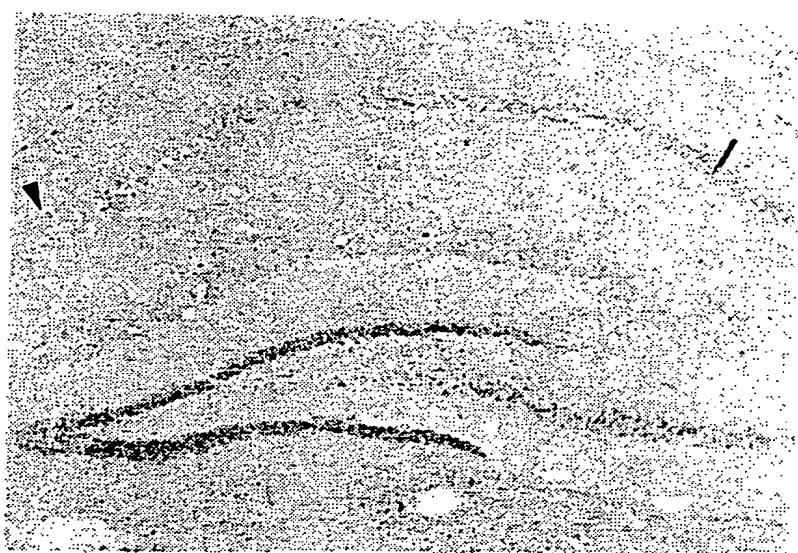


Fig. 10B

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Fig. 11B

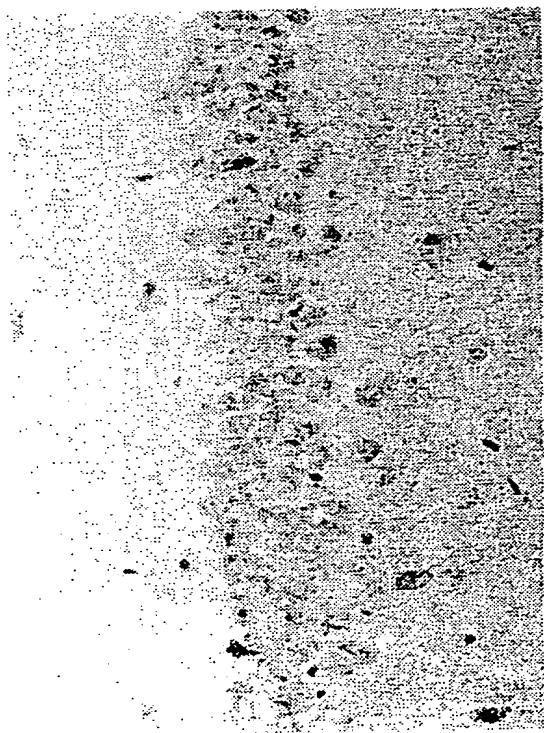


Fig. 11A



Fig. 11C



Fig. 11D



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ROSTRAL SECTIONS



Fig. 12A

CAUDAL SECTIONS



Fig. 12B

*111
+
excess
111

Fig. 12C

Fig. 12D

17A/21

ROSTRAL SECTIONS



CAUDAL SECTIONS



Fig. 12E Fig. 12F

*183
+
excess
183

Fig. 12G

Fig. 12H



Fig. 13B



Fig. 13A



Fig. 13C

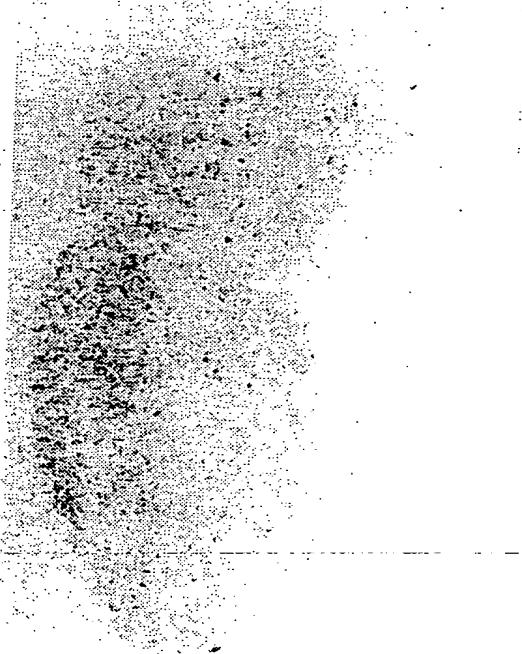


Fig. 13D

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	1	5	10	15	d	20	e	25	f	30
I.	a	b	c							
MVIIA	C K G K G A K	C S R	L M Y D C C T G S C	- R	- S G K - C					
MVIIIB	C K G K G A S	C H R	T S Y D C C T G S C N	R	- G K - C					
II.	s	t								
GVIA	C K S X G S S C S	X T S Y N C C R	- S C N X Y T	- K R C - - Y						
TVIA	C L S X G S S C S	X T S Y N C C R	- S C N X Y S R K - C R							
SNX-	C U S X G S S C S	R L M Y N C C R	- S C N X Y S R K - C R							
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III.	RVIA	C K P X G S X C R V S S Y N C C S	- S C K S Y - N K K C G							
SVIA	C R S S G S X C G V T S I	- C C - G R C - - Y R G K - C T								
SVIB	C K L K G Q S C R K T S Y D C C S G S C G R - S G K - C									
GVIIA	C K S X G T X C S R G M R D C C T	- S C L L Y S N K - C R R Y D								
SNX-	C K S T G S S C S R L M Y N C C R	- S C N T Y S R K - C R								
SNX-	C K S T G S S C S R L M Y N C C R	- S C N T Y S A K - C R								
	218									

MVIIIC/
SNX- C K G K G A P C R K T M Y D C C S G S C G R - R G K - C
230

Fig. 14

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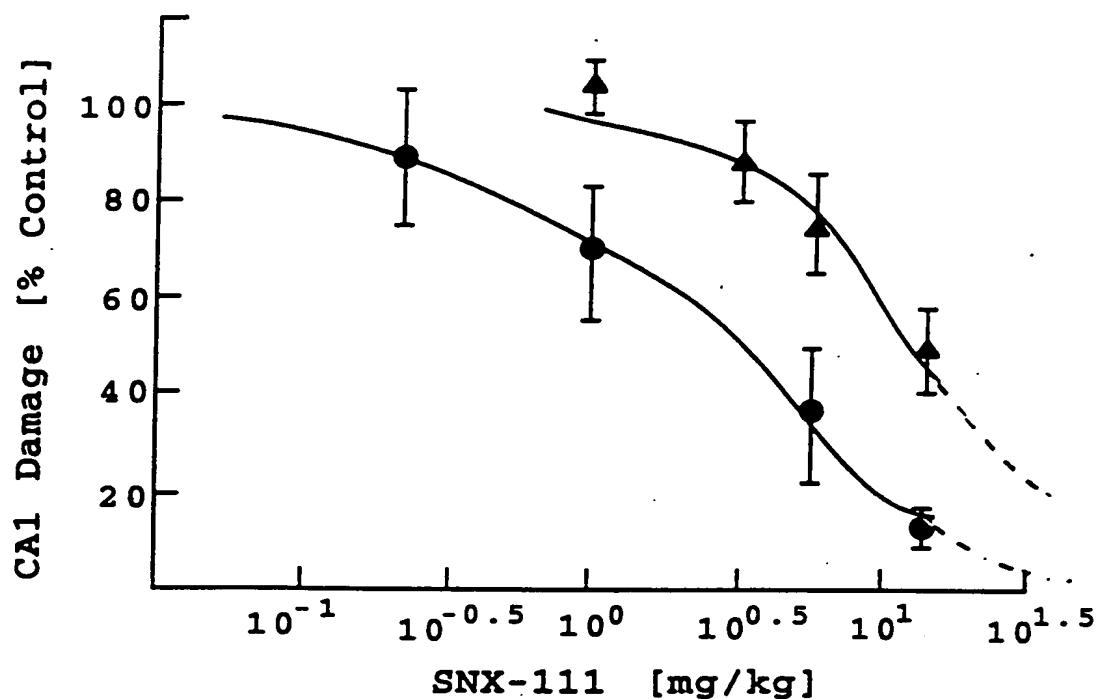


Fig. 15

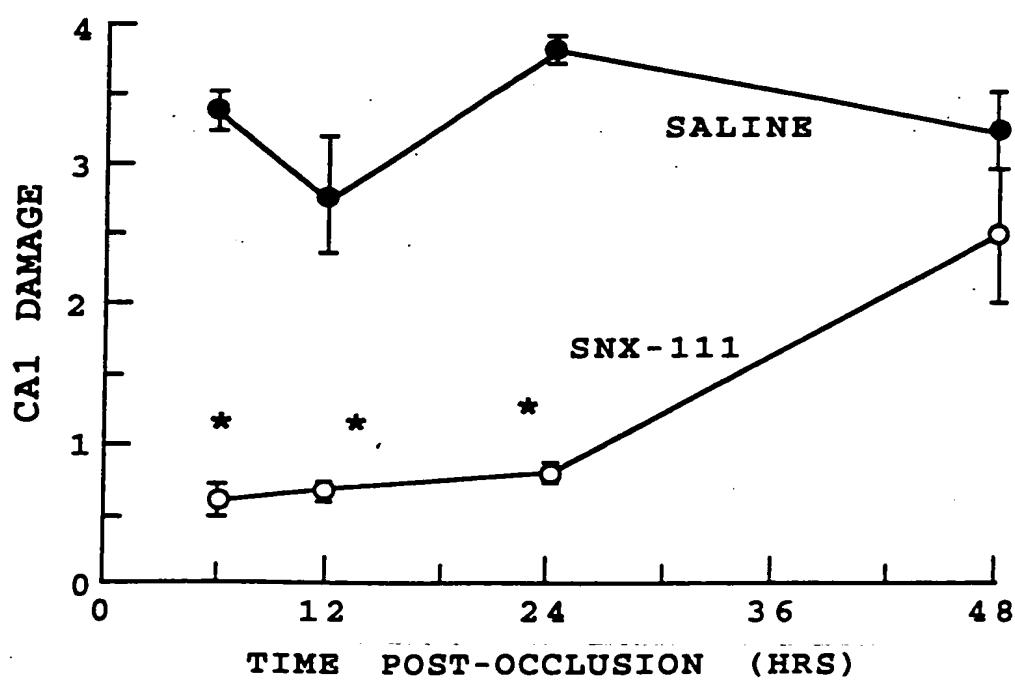


Fig. 16

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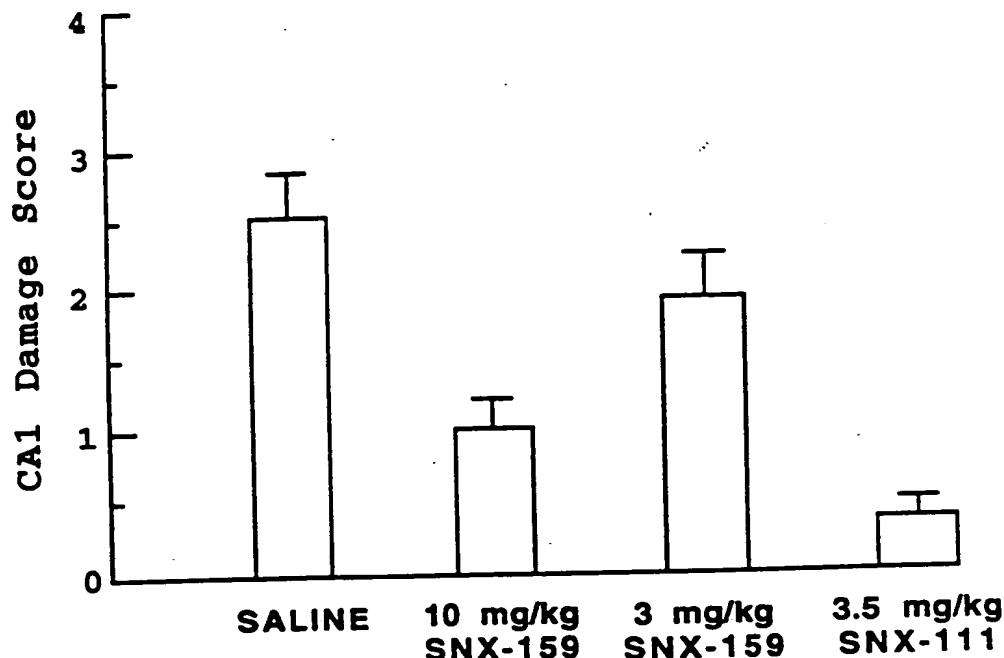


Fig. 17

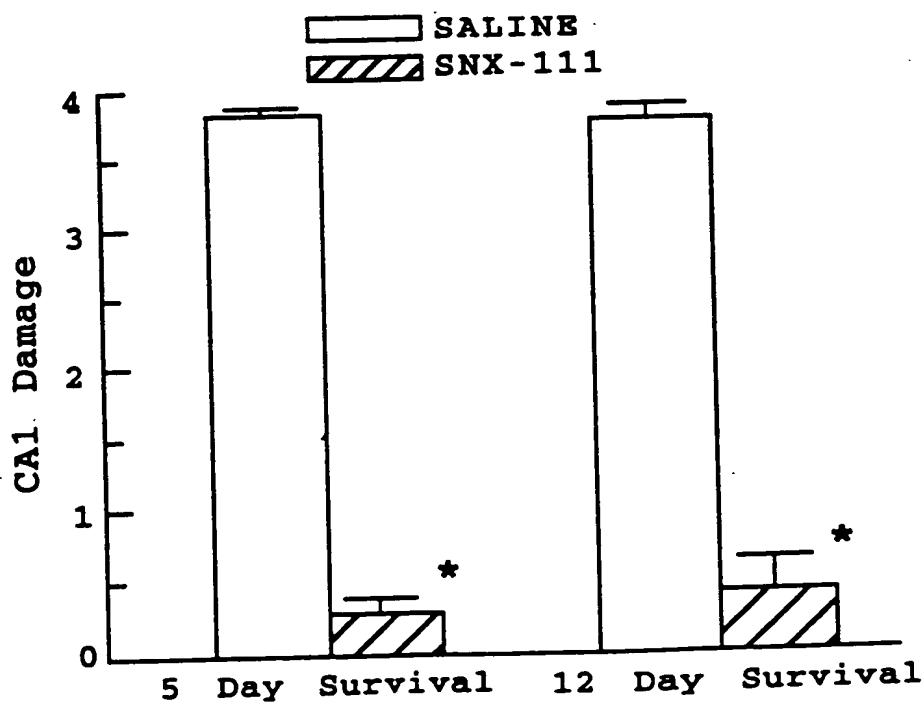


Fig. 18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/09766

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-8 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compounds.

Furthermore claims 1-6 were searched in part regarding the type of com -

2. Claims Nos.:

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

pound. Because the description lacks any further information the search was restricted to conotoxins.

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9209766
SA 67257

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 15/03/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9107980	13-06-91	US-A-	5051403	24-09-91
		AU-A-	6964091	26-06-91

INTERNATIONAL SEARCH REPORT
International Application No.

PCT/US 92/09766

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 C07K7/10; A61K37/02

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 107 980 (NEUREX CORPORATION) 13 June 1991 cited in the application see detailed description pages 8-38 see claims 1-3,5-10,13-16; examples ---	1-15
X	BIOCHEMISTRY vol. 26, no. 8, 21 April 1987, EASTON, PA US pages 2086 - 2090 B.M. OLIVEIRA ET AL. 'Neuronal Calcium Channel Antagonists. Discrimination between Calcium Channel Subtypes Using omega-Conotoxin from Conus magus Venom' see discussion see figure 1 ---	3-8,15 -/-

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- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

1 Date of the Actual Completion of the International Search
15 MARCH 1993

Date of Mailing of this International Search Report
29. 03. 93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

FUHR C.K.B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 84, June 1987, WASHINGTON US pages 4327 - 4331 E.W. MCCLESKEY ET AL. 'omega-Conotoxin: Direct and persistent blockade of specific types of calcium channels in neurons but not muscle' cited in the application see discussion</p> <p>-----</p>	3-8

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